Re-thinking the Role of Ribosomal Proteins in the Mdm2-p53 Axis

Lilyn Daftuar

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2013

ABSTRACT

Re-thinking the Role of Ribosomal Proteins in the Mdm2-p53 Axis

Lilyn Daftuar

The Mdm2-p53 axis is an important pathway in cells that is frequently misregulated in cancer. Under basal conditions, Mdm2 suppresses p53 through multiple mechanisms. However, when stress is encountered, this suppression is lifted and p53 transactivates the expression of many target genes to effect outcomes such as cell cycle arrest and apoptosis. One type of stress that can activate p53 is ribosomal stress, also called nucleolar stress. Ribosomal stress occurs when mishaps occur in ribosomal biogenesis, and various ribosomal proteins (RPs) have been shown to signal to Mdm2 and activate p53. This thesis presents two studies in the regulation of the Mdm2-p53 axis by ribosomal proteins.

In the first study, three ribosomal proteins are newly linked to the Mdm2-p53 axis. RPL37, RPS15, and RPS20 are shown to bind to Mdm2, inhibit its E3 ubiquitin ligase activity towards itself and p53, upregulate various p53 target genes, and cause both G2 arrest and apoptosis. Additionally, they downregulate levels of MdmX, a homolog of Mdm2 that also suppresses p53 activity.

In the second study, a novel extra-ribosomal function has been identified for RPL36A. Unlike other ribosomal proteins that interact with and activate the Mdm2-p53 axis, RPL36A represses it. RPL36A enhances the E3 ubiquitin ligase

activity of Mdm2, downregulates p53 levels, and inhibits the response to ribosomal stress.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Carol Prives, for providing me with valuable guidance and support throughout the years. She has taught me to think critically and created a wonderful lab environment for learning and research. Her scientific wisdom and enthusiasm for research are extraordinary.

I would also like to thank the members of the Prives lab, particularly Dr. Yan Zhu for her insightful feedback to my endless questions over the years. She has been a true teacher and is an inspiration.

Finally, I am thankful to my family and friends who have provided me with much needed emotional support, particularly my husband Sumit Daftuar.

PREFACE

This thesis is divided into four chapters. Chapter one is an introduction to the Mdm2-p53-MdmX network, ribosomes and ribosomal proteins (RPs), and their intersection. Chapter two is modified from a manuscript published in PLOS ONE entitled “Ribosomal proteins RPL37, RPS15, and RPS20 regulate the Mdm2-p53-MdmX network.” Chapter three is a manuscript in preparation describing the regulation of the p53-Mdm2 axis by RPL36A. Chapter four discusses future directions for the RP-Mdm2-p53 field.

CHAPTER 1

INTRODUCTION

THE MDM2-P53-MDMX NETWORK

Discovery of p53 and its importance

When thinking of cancer, a disease characterized by uncontrolled cellular proliferation, one must consider p53, the so-called “guardian of the genome (Lane, 1992). p53 is an important tumor suppressor in cells, and its loss or mutation has been implicated in at least half of all cases of human cancers (Levine and Oren, 2009). The earliest reports showed its locus on the short arm of chromosome 17 was lost in 75% of cases of colorectal carcinoma (Vogelstein et al., 1988) and the gene was mutated in a similar proportion of lung cancer (Takahashi et al., 1989). In particular, many cancers show loss of heterozygosity in which the wild-type allele is deleted from its locus, and the other allele is expressed as a missense mutation (Baker et al., 1989; Nigro et al., 1989).

p53 was first discovered in 1979 (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979) as a protein that bound to the large T antigen of Simian vacuolating virus 40 (SV40), a DNA virus that is present in primate populations and has been shown to cause cancer in hamsters (Cicala et al., 1993). p53 was so named because it runs at 53kD on an SDS-PAGE gel, and it was initially thought of as an oncogene in part because early experiments showed it was highly expressed in SV40-transformed cells but not

non-transformed cells, and it could cooperate with oncogenes such as Ras to transform cells (DeLeo et al., 1979; Eliyahu et al., 1984; Parada et al., 1984). However, it was later determined that the early experiments were performed using a mutant form of p53 that had acquired oncogenic properties, and wildtype p53 functions as a tumor suppressor (Finlay et al., 1989; Hinds et al., 1989). (After its gene was cloned, its sequence revealed that p53 has a molecular mass of only 44kD.)

Supporting its importance as a tumor suppressor, p53 is targeted for inactivation by all known DNA tumor viruses such as SV40, human papillomavirus (HPV), and Epstein-Barr virus (EBV) (Collot-Teixeira et al., 2004). Cancer also frequently arises spontaneously in the absence of viral infection, and further evidence for the importance of p53 in cancer comes from the study of Li-Fraumeni, a human genetic disorder. Li-Fraumeni patients inherit a defective allele of p53 and are extremely cancer-prone (Malkin et al., 1990; Srivastava et al., 1990), as are mice engineered to carry mutant p53 genes (Lavigueur et al., 1989) and knockout mice that lack both copies of p53 (Donehower et al., 1992).

Further underlining the importance of p53, knock-in mice carrying a hyper-active form of p53 are protected from cancer (Tyner et al., 2002), as are mice with an increased genetic dose of p53 (Garcia-Cao et al., 2002). Furthermore, restoring p53 function in various mouse models leads to tumor regression (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). It should be noted that unlike other tumor suppressors that are often deleted in cancer, p53 is

frequently mutated. Mutant forms of p53 can function as a dominant-negative, interfering with wildtype p53 function, or can function as a gain-of-function mutation and exert novel oncogenic effects (Brosh and Rotter, 2009).

Structure of p53 and its functions in cells

p53 and its family members p63 and p73 are highly conserved among vertebrates (Lane et al., 2011; Lu et al., 2009), and p53 homologs have been identified in several invertebrate species including placozoans, arachnids, nematodes, and flies (Lane et al., 2010a; Lane et al., 2010b; Rutkowski et al., 2010). Human p53 is a 393 amino acid protein that consists of a N-terminal transactivation domain (TAD1, residues 1-40, TAD2 residues 41-61) and a proline-rich region (PRR, residues 62-93). The central region of p53 contains a DNA binding domain (DBD, residues 94-292), a linker region (residues 293-324) harboring a nuclear localization signal (NLS), and a tetramerization domain (TET, residues 325-356) harboring a nuclear export signal (NES). Finally, the C-terminus of p53 consists of a C-terminal regulatory domain (CTD, residues 357-393) that harbors two additional NLSs (Figure 1.1).

The N-terminus and the C-terminus of p53 are unstructured (Dawson et al., 2003), as is the linker region separating the DBD from the TET domain. Thus, while the crystal structures of both the TET domain and the DBD have been solved down to the atomic level (Chen et al., 2010; Cho et al., 1994; Jeffrey et al., 1995; Kitayner et al., 2006), the crystal structures of the ends of the protein,

and of the entire protein, remain elusive. However, the N-terminus can be stabilized and crystalized if bound to RPA (Bochkareva et al., 2005), as can the C-terminus if bound to 14-3-3 (Schumacher et al., 2010). Additionally, both the N-terminus and the entire protein have been visualized using nuclear magnetic resonance (NMR) imaging (Tidow et al., 2007; Wells et al., 2008) or cryo-electron microscopy (Okorokov et al., 2006).

In the cell, p53 acts as a stress-responsive sequence-specific transcription factor that stimulates or downregulates expression of numerous target genes (Beckerman and Prives, 2010; Laptenko and Prives, 2006). The DBD of p53 recognizes a symmetrical 20 base pair consensus sequence in target genes: a 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' 10-mer that is separated from a second 10-mer by 0-13 base pairs (el-Deiry et al., 1992; Funk et al., 1992; Riley et al., 2008). Each half-site is bound by two p53 molecules, and the binding of a dimer of dimers is necessary to induce target gene transcription (Ho et al., 2006; Joerger and Fersht, 2008; Kitayner et al., 2006; Klein et al., 2001; Malecka et al., 2009; Rippin et al., 2002). Perhaps three of the most well studied p53 targets are Mdm2, p21, and Puma, each associated with different cellular outcomes upon activation by p53. As described below, Mdm2 is important for downregulating p53 levels and activity, thus the induction of Mdm2 by p53 creates an important negative feedback loop for shutting down p53 activity in cells. p21 (also known as Waf1, Cip1, or Cdkn1a) plays an important role in arresting the cell cycle and promoting senescence, and Puma is a pro-apoptotic protein.

p21 binds to CDK2 and inhibits it from phosphorylating Rb. As a result, E2Fs remain sequestered and are unable to commence DNA replication (Nevins, 2001). p21 also binds to proliferating cell nuclear antigen (PCNA) and prevents it from interacting with DNA polymerase delta during DNA replication and repair (Cayrol et al., 1998). Both mechanisms allow for efficient arrest in the G1 phase of the cell cycle, although p21 can also stimulate arrest in other parts of the cell cycle by inhibiting the activity of other CDKs. It should also be noted that p21 can be activated through p53-independent pathways (Abbas and Dutta, 2009).

Puma induces apoptosis by binding to and inhibiting the anti-apoptotic protein Bcl2 and its family members (Nakano and Vousden, 2001). This releases the pro-apoptotic proteins Bax and Bak, which stimulate permeabilization of the mitochondrial outer membrane and release of cytochrome C (Chipuk and Green, 2008). p53 can also directly induce the expression of Bax (Miyashita et al., 1994) or the pro-apoptotic Bcl2 family member Noxa (Oda et al., 2000; Shibue et al., 2003), and has multiple other mechanisms to stimulate apoptosis (Chipuk and Green, 2006).

Besides autoregulation, cell cycle arrest, and apoptosis, p53 also plays additional roles in DNA repair, metabolism, autophagy, embryo implantation, and more (Vousden and Prives, 2009) by stimulating the expression of target genes such as p53R2 (Tanaka et al., 2000), Sco2 (Matoba et al., 2006), Tigar (Bensaad et al., 2006), Dram (Crighton et al., 2006), and Lif (Hu et al., 2007). p53 also has non-transcription based mechanisms for affecting cell fate. For example, p53 can

activate senescence by binding to and stabilizing the mRNA of plasminogen activator inhibitor-1 (Pai-1) (Kortlever and Bernards, 2006; Shetty et al., 2008). Furthermore, cytoplasmic p53 can stimulate apoptosis by promoting the oligomerization of the pro-apoptotic protein Bax at the mitochondrial membrane (Baptiste and Prives, 2004; Chipuk et al., 2004; Erster et al., 2004).

Regulation of p53 by Mdm2 and MdmX

Given its importance in cell cycle arrest and apoptosis and other cellular outcomes, p53 is tightly regulated by Mdm2 (which stands for “Mouse Double Minute 2” and is also known as Hdm2) and its closely related homolog MdmX (also known as Mdm4 or HdmX). Both Mdm’s are RING (which stands for “Really Interesting New Gene”) domain-containing proteins that are highly conserved among vertebrates, and an Mdm gene has also been identified in invertebrates (Lane et al., 2011; Lane and Verma, 2012; Momand et al., 2011).

Human Mdm2 is a 491 amino acid protein with distinct domains. It contains a N-terminal p53-binding domain (residues 18-101) that has been crystallized in complex with p53 (Kussie et al., 1996) and observed alone by NMR (Uhrinova et al., 2005). The N-terminus is separated from a central acidic domain (AD, residues 237-288) by a flexible linker that contains both a NLS and a NES. Adjacent to the AD is a Zinc finger domain (residues 289-331) whose structure has been determined by NMR (Yu et al., 2006). At the C-terminus of Mdm2 lies a nucleolar localization signal (NoLS)-containing RING domain

(residues 437-482). MdmX is similar in size and structure to Mdm2 at 490 amino acids, although it lacks NLS, NES, and NoLS sequences (Figure 1.2). Mdm2 and MdmX interact with each other through their RING domains (Tanimura et al., 1999), and the crystal structures of the two RING domains bound together has been solved (Linke et al., 2008). But it is important to note that while the RING domain of Mdm2 functions as an active E3 ubiquitin ligase, transferring ubiquitin onto lysines of target proteins, the RING domain of MdmX is inactive (Sharp et al., 1999).

The N-termini of both Mdm2 and MdmX can bind to the N-terminus of p53 to inhibit its transactivation activity (Chen et al., 1993; Momand et al., 1992; Oliner et al., 1993). In the case of Mdm2, secondary sites of interaction have been identified between the central region of Mdm2 (residues 235-300) and p53 (Kulikov et al., 2006), and between the N-terminus of Mdm2 and the C-terminus of p53 (Poyurovsky et al., 2010).

Mdm2 has additional well-established mechanisms for regulating p53 besides inhibiting its transactivation activity. One group found that the RING domain of Mdm2 can bind to p53 mRNA and suppress its translation (Candeias et al., 2008). But the most well studied function of the Mdm2 RING domain is to ubiquitinate the C-terminal domain of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), although both the acidic domain (Kawai et al., 2003b; Meulmeester et al., 2003) and extreme C-terminus (Poyurovsky et al., 2007; Uldrijan et al., 2007) also play essential roles. As with other ubiquitinated

species, p53 is degraded by the 26S proteasome when poly-ubiquitinated, but as a result of mono-ubiquitination by Mdm2, p53 is preferentially exported from the nucleus (Lohrum et al., 2001). In the cytoplasm, the formation of Mdm2-p300 or Mdm2-CBP E3-E4 complexes facilitates the efficient production of poly-ubiquitinated p53 species (Grossman et al., 2003; Shi et al., 2009). MdmX supports this function of Mdm2 by forming hetero-oligomers that stabilize it and direct its ubiquitination activity towards p53 (Stad et al., 2000); in the absence of MdmX, Mdm2 homo-oligomers form and preferentially trans-ubiquitinate and autodegrade (Wade et al., 2010).

While the cell contains other E3 ubiquitin ligases that can ubiquitinate p53 (Jain and Barton, 2010), studies in mice demonstrate the importance of Mdm2 and MdmX in regulating p53. Mdm2 is essential for keeping p53 in check, since Mdm2-/- knockout mice are early embryonic lethals while p53-/- Mdm2-/- double knockout mice survive (Jones et al., 1995; Montes de Oca Luna et al., 1995). Similarly, MdmX-/- knockout mice die *in utero* (Finch et al., 2002), while p53-/- MdmX-/- double knockout mice survive (Parant et al., 2001), indicating the importance of both Mdm's in regulating p53 during embryonic development. The importance of both Mdm2 and Mdm2 in regulating p53 in adult tissues has also been explored using conditional knockout and knockin mice. Deletion of either Mdm2 or MdmX from neuronal progenitor cells or post-mitotic neurons leads to p53-dependent apoptosis (Francoz et al., 2006; Xiong et al., 2006). Similarly, if Mdm2 or MdmX is absent when p53 is transiently restored in an inducible p53-

ER mouse model, then unregulated p53 leads to death of the mouse or arrest of proliferating hematopoetic cells (Ringshausen et al., 2006; Garcia et al., 2011).

However, it should be noted that Mdm2 and MdmX exhibit p53-independent functions too (Manfredi, 2010; Wade et al., 2013). For example, Mdm2 downregulates p21 protein levels in an ubiquitin-independent manner (Jin et al., 2003), and if Mdm2 is knocked down by siRNA, p21 is synthesized but is unable to induce cell cycle arrest (Giono and Manfredi, 2007). Mdm2 also binds to Nbs1, a DNA repair protein, and inhibits repair of double strand breaks (Alt et al., 2005). Additionally, several mouse models suggest p53-independent roles exist for Mdm2 *in vivo*. If Mdm2 is overexpressed throughout a mouse, it can cause a unique variety of tumors to arise, different from the tumor pattern seen in p53-null mice (Jones et al., 1998). Mdm2 degradation targets outside of the Mdm2-p53-MdmX network may contribute to its p53-independent oncogenic functions. For example, Mdm2 can ubiquitinate and degrade Rb (Uchida et al., 2005), leading to cell cycle progression. Mdm2 can also target Numb for degradation (Juven-Gershon et al., 1998). Numb is involved in nervous system development system and is responsible for inhibiting Notch signaling in proliferating cells, so Mdm2-mediated degradation may inappropriately activate Notch signaling. In the case of MdmX, the data from mouse models is less clear. One study found overexpressing MdmX was not tumorigenic (De Clercq et al., 2010) while another study found it was tumorigenic (Xiong et al., 2010). Since MdmX can inhibit cellular proliferation by Interacting with and inhibiting the

transcription factor E2F1 (Strachan et al., 2003), perhaps its p53-independent anti-proliferative properties are more relevant *in vivo*.

Finally, it should be noted that, unlike p53, Mdm2 and MdmX are rarely mutated in human cancers; rather they are often amplified or overexpressed (Momand et al., 1998; Wade and Wahl, 2009). The few cases of mutant Mdm2 that have been identified are missense mutations within the central acidic region. As described below, the central acidic region of Mdm2 is also the site of interaction for various ribosomal proteins, and disrupting their interactions has implications *in vivo*.

Activation of p53 in response to stress

Thus, although p53 is continually produced in cells, its rapid ubiquitination and degradation means it has a short half-life and levels of it are kept low. But upon the introduction of stress, Mdm2 and p53 are modified so that Mdm2 cannot bind to it and target it for degradation. As a result, levels of p53 can rise rapidly and the cell can quickly respond to the stress (Figure 1.3). Cellular stress can come in many forms, the first of which to be identified was DNA damage induced by ultraviolet (UV) irradiation (Maltzman and Czyzyk, 1984). UV irradiation causes the formation of pyrimidine dimers that can be repaired by nucleotide excision repair (NER), while ionizing radiation (IR) causes double-strand breaks (DSBs) in DNA that can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) (Houtgraaf et al., 2006).

In HR, the MRN complex (consisting of Mre11, Rad50, and Nbs1) binds to the broken ends to stabilize them, and recruits ATM, an essential serine/threonine (S/T) kinase that is strongly tumorigenic when mutated (Barlow et al., 1996; Chun and Gatti, 2004) and whose kinase activity is necessary for maintaining genomic stability (Yamamoto et al., 2012). ATM facilitates DNA repair, cell cycle arrest and apoptosis by phosphorylating many targets including itself, Mre11, Nbs1, Sae2 and H2AX (Baroni et al., 2004; Burma et al., 2001; Lavin, 2007). Sae2 is the endonuclease response for resecting the broken DNA to generate single-stranded DNA that is capable of invading the homologous chromosome, the first step in HR (Symington and Gautier, 2011). ATM also phosphorylates Chk2, another S/T kinase, which goes on to phosphorylate p53 on S20 in the N-terminus (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000) and on S366, S378, and T387 in the C-terminus (Ou et al., 2005).

ATM also directly phosphorylates p53 on S15 (Canman et al., 1998) and S46 (Kodama et al., 2010), Mdm2 on both its N-terminus (Khosravi et al., 1999) and on S395 in the RING domain (Maya et al., 2001), and MdmX on S342, S367, and S403 (Chen et al., 2005). Other kinases can also phosphorylate p53 and Mdm2, such as DNA-PK, an S/T kinase involved in NHEJ (Mayo et al., 1997; Shieh et al., 1997). The effect of phosphorylated Mdm2 is to prevent it from interacting with and degrading p53 (Chen, 2012), and a recent study found phospho-Mdm2 can also bind to p53 mRNA to stimulate its translation (Gajjar et al., 2012). (This is in contrast to un-modified Mdm2 found in un-stressed cells,

where Mdm2 was found to bind to p53 mRNA and suppress its translation (Candeias et al., 2008).) Finally, after the stress has passed, p53 can shut itself down by upregulating levels of both Mdm2 and Wip1 (also known as Ppm1d), a phosphatase that can de-phosphorylate both p53 and Mdm2 (Fiscella et al., 1997; Lu et al., 2007; Takekawa et al., 2000).

DNA damage also affects MdmX levels and localization in a modification-dependent manner. Early studies found ectopic MdmX is predominantly cytoplasmic basally but re-localizes to the nucleus after DNA damage (Li et al., 2002). However, other studies have found that levels of endogenous MdmX drop in response to (non-lethal) stress due to an increase in Mdm2-mediated ubiquitination (Kawai et al., 2003a). Levels of MdmX may also drop in response to the p53-mediated upregulation of miR-34a, a micro RNA that can target various mRNAs, such as MdmX mRNA, for RISC-mediated degradation (Mandke et al., 2012; Markey and Berberich, 2008). As a result of the decreased levels of MdmX, the formation of Mdm2 homo-oligomers that trans-ubiquitinate may be favored, rather than the formation of Mdm2-MdmX hetero-oligomers that target p53 for degradation. In mice, if residues S342, S367, and S403 are mutated such that they can no longer be phosphorylated by ATM or Chk2 in response to DNA damage, then MdmX degradation is inhibited and p53 activation is attenuated (Wang et al., 2009). Similarly, if MdmX is overexpressed in mice, the mice exhibit a dampened p53 response to IR (Xiong et al., 2010). Interestingly, a fraction of

cellular MdmX associates with Bcl2 at mitochondria and can facilitate p53-mediated apoptosis in response to a lethal dose of UV (Mancini et al., 2009).

Besides phosphorylation by kinases, other binding partners can modify p53 and Mdm2 in response to DNA damage or other forms of stress. For example, p300 is an acetyl transferase that can bind the N-terminus of phospho-p53 (Meek and Anderson, 2009), acetylate p53 on K382 and other residues in response to UV or IR (Sakaguchi et al., 1998), and play an important role in inhibiting p53's interaction with Mdm2 and promoting p53 stability after stress (Ito et al., 2001; Tang et al., 2008). The C-terminus of p53 can also be modified by the addition of methyl, Nedd8, or Sumo moieties (Carter and Vousden, 2009), and various post-translational modifications to p53 may be associated with different cellular outcomes (Carvajal and Manfredi, 2013; Vousden and Prives, 2009). For example, modifications to the two transactivation domains within the N-terminus of p53 (TAD1 and TAD2) promote the transactivation of different target genes. When TAD1 is modified in response to DNA damage, p21 is induced and cell cycle arrest follows. On the other hand, modifications to TAD2 following oncogene activation tend to promote apoptosis (Brady et al., 2011).

Besides DNA damage, many other forms of stress can also activate p53, such as include hypoxia, heat shock, spindle damage, nutritional starvation, and more (Vousden and Prives, 2009). In the case of oncogenic stress, Arf (which stands for "Alternate Reading Frame") plays an important role. Arf is a nucleolar protein whose genetic locus overlaps with Ink4a, a CDK inhibitor that blocks Rb

phosphorylation and progression of the cell cycle beyond G1. The expression of the alternate reading frame of this locus, rather than Ink4a, is induced by activation oncogenes such as Ras or Myc, and Arf can interact with the central acidic domain of Mdm2 and sequester it in the nucleolus, allowing p53 to be activated (Weber et al., 1999; Zhang et al., 1998). Arf overexpression can also lead to the addition of Sumo moieties onto Mdm2, followed by inhibition of Mdm2 activity and accumulation of p53 (Xirodimas et al., 2002).

RIBOSOMES AND RIBOSOMAL PROTEINS

Ribosome structure and function

Ribosomes are highly conserved structures consisting of both proteins and nucleic acids responsible for protein synthesis in all living cells. The crystal structures of the 70S prokaryotic bacterial ribosome and the 80S eukaryotic yeast ribosome have been solved (Ban et al., 2000; Ben-Shem et al., 2011; Jenner et al., 2012; Mitra et al., 2005; Schluenzen et al., 2000; Schuwirth et al., 2005; Wimberly et al., 2000). The eukaryotic ribosome is significantly larger than the bacterial ribosome due to the increased length and number of strands of catalytic rRNA, as well as the introduction of 47 additional ribosomal proteins (RPs) that have no bacterial homolog. In the case of human ribosomes, the 40S small subunit consists of 33 proteins (RPS2 – RPS30) surrounding 1 strand of rRNA (18S), while the 60S large subunit consists of 46 proteins (RPL2 – RPL41) surrounding 3 strands of rRNA (5S, 5.8S, and 28S). In addition, mitochrondria have their own ribosomes with their own mitochondrial RPs (MRPs) and mitochondrial rRNAs organized into small and large subunits (Cavdar Koc et al., 2001; Koc et al., 2001). Of particular note to the rest of this thesis are the RPs outlined in Figure 1.4.

During protein synthesis, ribosomes translate mRNA into amino acids in a complex process (Dever and Green, 2012; Jackson et al., 2010). In the first step, the 40S small subunit recognizes mRNA and identifies the AUG start codon, combines with a methionine-charged tRNA, and recruits the 60S large subunit.

During elongation, the active 80S ribosome travels along mRNA in the 5'-3' direction, accepts charged tRNAs containing anticodons complementary to mRNA codon sequences, catalyzes peptide bond formation between the amine end of the newly accepted amino acid and the carboxy end of the growing polypeptide chain, and releases the emptied tRNA. While the rRNA component of ribosomes exhibit peptidyl transferase activity, the protein component of ribosomes drive major conformational changes after codon-anticodon matches have been produced. Finally, during termination, the stop codon is recognized, the nascent polypeptide is released, and the small and large ribosomal subunits separate in preparation for a new cycle of translation.

Ribosomal biogenesis

Ribosomal biogenesis occurs within the nucleolus of higher eukaryotes (Boisvert et al., 2007). Nucleoli are non-membrane bound compartments of nuclei that organize around clusters of rDNA genes during interphase. rDNA is transcribed into an immature 45S rRNA precursor by RNA polymerase I in the fibrillar center (FC) of the nucleolus, and snoRNPs such as nucleolin and fibrillarin process it into mature 5.8S, 18S, and 28S rRNAs in the dense fibrillar component (DFC) of the nucleolus. (5S rRNA is transcribed by RNA polymerase III in the nucleoplasm and then directed to the nucleolus for incorporation into nascent 60S large subunits.) Mature strands of rRNA then associate with ribosomal proteins (RPs) within the granular component (GC) of the nucleolus

with the help of proteins such as nucleophosmin (also known as NPM, B23, or numatrin). The assembled 40S small and 60S large subunits are finally exported from the nucleoplasm into the cytoplasm, and come together to form an active 80S ribosome upon recruitment by eukaryotic initiation factors (eIFs) (Jackson et al., 2010).

The genes encoding the RPs are transcribed into mRNA by RNA polymerase II, and in yeast, approximately 50% of RNA pol II transcripts are RP mRNAs (Warner, 1999). While RP mRNAs levels are carefully regulated in yeast and have short half-lives, RP mRNA levels vary and are relatively stable in higher eukaryotes (Perry, 2007). In both species, as with other protein-encoding genes, RP mRNAs are exported into the cytoplasm for translation. The translation of vertebrate RP mRNAs is carefully regulated by 5' terminal oligopyrimidine (5'-TOP) sequences that are targeted by growth pathways such as mTOR-SK61. The coordinate translation of RP mRNAs in response to mitogenic signals may help balance RP production and ensure the proper RP stoichiometry is achieved (Meyuhas, 2000).

After synthesis, RPs enter the nucleus and are targeted to the nucleolus, where they assemble with 18S and 5S / 5.8S / 28S rRNAs to form the 40S small and 60S large ribosomal subunits, respectively. While RPs that have been incorporated into ribosomal subunits are stable with a half-life on the order of days, excess unassembled RPs have a half-life on the order of minutes and are rapidly degraded in the nucleoplasm (Lam et al., 2007). Although perhaps

energetically wasteful, this assures RPs are abundant and their availability is not rate limiting for ribosome production and cellular growth. Additionally, this rapid degradation may prevent RPs, which are highly basic and bind strongly to nucleic acids, from interacting with nucleoplasmic components inappropriately (Warner, 1999).

However, GFP-RPs have also been shown to shuttle slowly between the nucleolus and nucleoplasm, with fluorescence recovery after photopbleaching (FRAP) taking a few minutes. Similarly, proteins involved in rRNA transcription and processing also shuttle between the nucleolus and nucleoplasm, but much more rapidly (Chen and Huang, 2001). These results may not be too surprising, since nucleoli are not bound by any membranes, so proteins ought to be able to diffuse freely between nucleoli and the nucleoplasm. Presumably, only nascent RPs not yet bound to rRNA and not yet degraded can exhibit this movement, while RPs that have been incorporated into 40S or 60S ribosomal subunits cannot. Alternatively, some excess RPs may linger in the nucleoplasm and serve extra-ribosomal functions (Komili et al., 2007; Schroder and Moore, 2005; Wool, 1996).

Roles for ribosomal proteins beyond the ribosome

While ribosomal proteins (RPs) are well understood in terms of their contribution to ribosome structure and function, there are increasing lines of evidence that some RPs have extra-ribosomal functions (Warner and McIntosh,

2009). Several RPs can auto-regulate their own mRNA or influence RNA polymerase III transcription of tRNA and 5S rRNA. For example, yeast RPL2 and RPS28 both have been shown to regulate their own mRNA levels (Badis et al., 2004; Presutti et al., 1991), and RPL30 and RPS14 can control splicing of their own mRNA (Eng and Warner, 1991; Fewell and Woolford, 1999). More recently, human RPS13 was also shown to bind its own mRNA and inhibit its splicing (Malygin et al., 2007), and RPS3 was shown to bind its own mRNA and inhibit its translation (Kim et al., 2010). Additionally, RPL6 can enhance RNA pol III activity *in vitro* (Dieci et al., 1993) and overexpressing it in yeast suppresses the growth defect caused by a mutant TFIIIC component (Dieci et al., 2009).

And while not an extra-ribosomal function *per se*, it is interesting that the two genetic loci for ubiquitin in eukaryotic cells is the same locus as two RP genes, RPS27A and RPL40 (Catic and Ploegh, 2005; Finley et al., 1989). Ubiquitin plays an important role in targeting proteins for proteasome-mediated degradation (Hochstrasser, 1996), and like RPs, it is highly conserved, abundant, and widely expressed. Ubiquitin is co-transcribed and co-translated with either one of the RPs, and the joined precursor protein is separated immediately after translation.

There are additional examples of RPs functioning outside of the ribosome and far afield from ribosomal biogenesis. Missense mutations were identified in RPL10 in two cases of autism (Klauck et al., 2006), and RPL10 has been found to bind to presenilin-1 (PS1) and c-jun in neuronal tissues (Imafuku et al., 1999).

RPL22 was also shown to bind to histone H1 in flies (Ni et al., 2006), although the significance of these protein interactions remains unclear. More recently, RPL13A was shown to be phosphorylated in response to interferon-gamma (IFNg) treatment of U937 cells. Phospho-RPL13A leaves the ribosome to associate with the GAIT complex (Mazumder et al., 2003; Mukhopadhyay et al., 2008) and reduce translation of specific target mRNAs such as ceruloplasmin and VEGF-A (Ray and Fox, 2007). Interestingly, despite the removal of RPL13A from ribosomes, protein translation appears to function normally.

From the small ribosomal subunit, RPS3 was shown to contain a biochemically active endonuclease domain (Kim et al., 1995), although its role in DNA repair in *vivo* remains unclear. RPS3 has also been shown to interact with NFkB in response to TNF stimulation and relocate to the nucleoplasm (Wan et al., 2007), and interact with both Mdm2 and p53 in the nucleoplasm in response to oxidative stress (Yadavilli et al., 2009). Additionally, RPS26 was recently identified as a type 1 diabetes susceptibility gene in a genome-wide SNP association study (Schadt et al., 2008).

Finally, a few RPs can directly regulate cellular growth. RPL23 was shown to bind to NPM, a nucleolar protein that helps rRNA and RPs assemble into small and large subunits, and prevent it from activating Miz1, an antagonist of the transcription factor Myc. Therefore, RPL23 can indirectly promote Myc activation and cellular proliferation (Wanzel et al., 2008). On the other hand, RPL11 and

RPS14 have been shown to bind to and inhibit the transactivation activity of Myc (Dai et al., 2007; Dai et al., 2010; Zhou et al., 2013b). RPL11 was further shown to downregulate Myc mRNA levels via micro-RNA silencing in response to ribosomal stress (Challagundla et al., 2011).

Ribosomal proteins and cancer

The importance of ribosomal biogenesis in cancer is perhaps suggested by the role of Arf, a tumor suppressor found in the nucleolus, in regulating it. Arf can suppress ribosomal subunit assembly by binding to NPM and inhibiting its function (Bertwistle et al., 2004). It can also suppress rRNA synthesis by binding to the RNA pol I transcription termination factor TTF1 and excluding it from the nucleolus (Lessard et al., 2010). When Arf is absent, cells have enlarged nucleoli and show increased ribosomal biogenesis (Apicelli et al., 2008).

But more direct links between ribosomal proteins (RPs) and cancer exist. For example, relevant to the RPs that will be discussed in the remainder this thesis, levels of RPS15, RPS27A, RPL26, and RPL37 (among several dozen other RPs) are elevated in colorectal cancer (Lai and Xu, 2007), and more recently, a link between overexpression of RPS20 and adverse outcome in medulloblastoma has been reported (De Bortoli et al., 2006).

Presumably, all RPs must be overexpressed in cancer simply to fuel the demands of rapidly growing cancer cells for increased polypeptide synthesis, and overexpressed RPs may contribute to a positive feedback loop where they

increase expression of proto-oncogenes aberrantly. For example, Akt can signal to mTOR to augment ribosomal biogenesis, and Myc can upregulate multiple targets including several RPs, RP processing proteins, and eIFs (Ruggero and Pandolfi, 2003). Indeed, pathologists have long observed enlarged or increased numbers of nucleoli in cancerous cells (Montanaro et al., 2008), and this "nucleolar hypertrophy" can serve as a prognostic marker in the clinic (Derenzini et al., 2009). Nevertheless, it is impossible to use correlations such as these to conclude a causative role exists for RPs in the development or prognosis of cancer.

For many model organisms, loss of even one allele of several RP genes impairs growth. A "Minute" phenotype has been observed in mice heterozygous for mutant RPS19 or RPS20 (McGowan et al., 2008), flies carrying mutant RPS3 (Saeboe-Larssen et al., 1998), and even plants whose levels of RPL23A were reduced used siRNA (Degenhardt and Bonham-Smith, 2008). In yeast, deletion of a single allele of RPS6 led to both a reduction in cell size and reduced growth rates (Chiocchetti et al., 2007).

More persuasive evidence for a causative role for RPs in cancer comes from other studies. One study suggested RPS3A may function as an oncogene, since overexpressing it in 3T3 cells led to their transformation, and overexpressing it in nude mice led to tumor formation (Naora et al., 1998). On the other hand, a zebrafish genetic screen for essential haploinsufficient tumor suppressors uncovered 11 RP genes, including RPS7, RPS15, and RPL36A

(Amsterdam et al., 2004). Additionally, RPS6 was identified as a tumor suppressor in flies, since silencing its expression led to the formation of tumors and abnormal hematopoetic cell differentiation (Watson et al., 1992). This result could not be recapitulated in mice, since knocking out even one allele of RPS6 resulted in embryonic lethality (Panic et al., 2006). However, eliminating its expression in the liver led to the cessation of hepatocellular division (Volarevic et al., 2000). Another study in mice identified a role for RPS19 and RPS20 in erythroid proliferation, since mutation of either one led to a reduction in the number of red blood cells (McGowan et al., 2008).

Finally, in humans, Diamond-Blackfan anemia (DBA), an autosomal dominant inherited disorder characterized by increased apoptosis of erythroid cells and a predisposition to cancer (Ellis and Lipton, 2008), is caused by mutations to RP genes. The most common mutation is to RPS19, but rarer cases have been reported where RPs such as RPS7, RPS15, RPS26, RPL5, or RPL11 are mutated (Badhai et al., 2009; Doherty et al., 2010; Gazda et al., 2008). Another human cancer-predisposition disease, 5q- syndrome, has been linked to mutations in RPS14 (Ebert et al., 2008). Additionally, aberrations in other aspects of ribosome function are also associated with cancer. Syndromes caused by mutation to rDNA production or rRNA processing genes include Cartilage-hair hypoplasia syndrome, Dyskeratosis congenita, Shwachman–Bodian–Diamond syndrome, and Treacher-Collins syndrome, and are also associated with defects in blood cell development and a predisposition to cancer (Ellis and Lipton, 2008).

It should be noted that siRNA-mediated depletion of any of the RPs involved in DBA has been shown to lead to a decrease in levels of all of the RPs of the same ribosomal subunit, a decrease in levels of that ribosomal subunit, and reduced levels of fully assembled ribosomes and polysomes (Robledo et al., 2008). Since this inhibition of ribosome function by knockdown of a RP leads to defects in cellular growth, it remains unclear how haploinsufficiency of a RP gene contributes to tumorigenesis in animals and suggests that the mechanisms by which RPs cause cancer may lie outside their canonical roles in the ribosome.

RIBOSOMAL PROTEINS AND MDM2

Several ribosomal proteins interact with Mdm2 and regulate p53

p53 can inhibit ribosomal biogenesis through multiple mechanisms (Budde and Grummt, 1999; Zhai and Comai, 2000), and loss of p53 function has been correlated with an upregulation in ribosome biogenesis (Montanaro et al., 2008). Conversely, overexpression of various ribosomal proteins (RPs) has been linked to an upregulation in p53 levels. The first of these to be described was RPL11, which can bind to Mdm2 and inhibit ubiquitination of p53 (Dai et al., 2006; Lohrum et al., 2003; Zhang et al., 2003). Although a physical interaction between Mdm2, p53, 5S rRNA, and RPL5 was identified prior to the RPL11-Mdm2 interaction (Marechal et al., 1994), its significance was unclear for another 10 years until it was shown that RPL5 can also inhibit Mdm2-mediated ubiquitination and degradation of p53 (Dai and Lu, 2004). Since then, multiple other RPs have also been shown to stabilize p53. These include RPL23 (Dai et al., 2004; Jin et al., 2004), RPL26 (Zhang et al., 2010), RPS7 (Chen et al., 2007; Zhu et al., 2009), RPS14 (Zhou et al., 2013a), RPS25 (Zhang et al., 2013), RPS26 (Cui et al., 2013), RPS27, RPS27A and RPS27L (Sun et al., 2011; Xiong et al., 2011).

All 11 of the RPs described above bind to the central region of Mdm2, and many of them can increase Mdm2 levels in addition to p53, although in several cases, it is unclear if they do so via p53 transcription (Figure 1.5). But a few clear differences between the RPs have been observed. For example, RPL26 has additional mechanisms for upregulating p53 levels besides inhibiting Mdm2-

mediated ubiquitination. RPL26 binds to both the 5' UTR and the 3' UTR of p53 mRNA to stimulate its translation after cells have been treated with ionizing radiation (Chen and Kastan, 2010; Takagi et al., 2005). When the cell is un-stressed, RPL26 is ubiquitinated by Mdm2 and targeted for degradation, and is inhibited from interacting with p53 mRNA by both Mdm2 (Ofir-Rosenfeld et al., 2008) and nucleolin (Chen et al., 2012).

Other ribosomal proteins are also modified by Mdm2, although under varying conditions and with various consequences. For example, like RPL26, Mdm2-mediated ubiquitination of RPS27L leads to its degradation (Xiong et al., 2011), while Mdm2 only ubiquitinates and degrades RPS27A in response to actinomycin D (ActD)-induced ribosomal stress (Sun et al., 2011). On the other hand, Mdm2-mediated neddylation of RPL11 can protect it from degradation (Sundqvist et al., 2009). In the case of RPS7, Mdm2-mediated ubiquitination does not lead to its degradation. Rather, ubiquitinated RPS7 can no longer inhibit Mdm2 auto-ubiquitination but can still selectively inhibit the degradation of p53, creating a feed-forward loop whereby Mdm2 levels drop but p53 accumulates (Zhu et al., 2009).

Ribosomal proteins can also be differentiated from each other based upon their effect on MdmX or their status as p53 target genes. RPS14 does not interact with MdmX and has no effect on its levels (Zhou et al., 2013a), while overexpression of RPS25 stabilizes MdmX (Zhang et al., 2013). On the other hand, RPL11 destabilizes MdmX by enhancing Mdm2-mediated ubiquitination

(Gilkes et al., 2006). Interestingly, 5S rRNA was recently shown to be involved in MdmX stability (Li and Gu, 2011). Finally, transcription of RPS27 is repressed by p53, while RPS27L transcription is enhanced (Xiong et al., 2011). Transcription of RPS25 is also repressed by p53 in response to ribosomal stress (Zhang et al., 2013).

Ribosomal proteins signal to p53 following ribosomal stress

Ribosomal biogenesis is an energetically expensive procedure for the cell. A mammalian cell spends approximately half of its resources on the creation of new ribosomes (Warner, 1999), so it makes sense for the cell to have evolved surveillance mechanisms to ensure its fidelity. Ribosomal stress, also known as nucleolar stress, occurs when mishaps occur in ribosomal biogenesis. These mishaps can arise from an inhibition to rRNA production or processing, an imbalance in RP levels, or an impairment to ribosomal subunit assembly (Boulon et al., 2010). Other types of stress that activate p53, such as DNA damage, oncogene activation or nutrient deprivation, have also been shown to trigger disruptions in nucleolar function (Rubbi and Milner, 2003). As a result, nucleoli may disintegrate and nucleolar proteins and RPs may relocalize into the nucleoplasm (Chen and Huang, 2001) and transduce ribosomal stress signals to p53. Mdm2 may also relocalize to nucleoli following ribosomal stress (Mekhail et al., 2005) and promote the ubiuqitination of p53 there (Boyd et al., 2011).

Ribosomal stress can be induced in cells by the application of actinomycin D (ActD), 5-fluorouracil (5FU), or mycophenolic acid (MPA). In the case of ActD, a low dose interferes with RNA pol I transcription of rDNA (Perry and Kelley, 1970), while 5FU can be incorporated into rRNA molecules and inhibit rRNA processing (Ghoshal and Jacob, 1994). MPA functions by inhibiting guanine nucleotide synthesis, leading to a block in rRNA transcription (Sun et al., 2008). In each of these cases, p53 levels are upregulated and cell cycle arrest or apoptosis is induced (Figure 1.6). Interestingly, several other drugs that are not thought of as ribosomal stress agents, such as cisplatin, doxorubicin, and MG132, can also disrupt transcription or rDNA or processing of rRNA (Burger et al., 2010).

For each the 11 aforementioned RPs that can upregulate p53 levels when overexpressed (RPL5, RPL11, RPL23, RPL26, RPS7, RPS14, RPS25, RPS26, RPS27, RPS27A, RPS27L), their knockdown by siRNA generally leads to a decrease in basal p53 levels (the few exceptions are siRPL23, siRPS14, and siRPS26, which upregulate basal p53 levels). Additionally, each of these RPs is necessary for a full response to ribosomal stress. When ActD is applied, levels of p53 are lower in siRPL5, siRPL11, siRPL23, siRPS7, siRPS14, siRPS27A, or siRPS27L cells than in siControl cells, and p21 upregulation is attenuated as well (Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004; Sun et al., 2011; Xiong et al., 2011; Zhou et al., 2013a; Zhu et al., 2009). In the case of siRPS25, p21 levels were not determined, but p53 and Mdm2 levels were attenuated following ActD-

induced ribosomal stress (Zhang et al., 2013). Interestingly, levels of p53 protein were not affected by ActD in a siRPS26 background, but both Mdm2 and p21 levels were decreased (Cui et al., 2013). (The ribosomal stress response in siRPL26 and siRPS27 cells has not been determined.)

But in other cases, knockdown of a RP induces p53 rather than suppressing its activation. For example, siRNA directed against RPL7A, RPL22, RPL24, RPL29, RPL30, RPL37, RPS6, RPS9, and RPS23 all lead to increased levels of p53 (Anderson et al., 2007; Barkic et al., 2009; Fumagalli et al., 2009; Lindstrom and Nister, 2010; Llanos and Serrano, 2010; Sun et al., 2010). In these latter cases, decreasing the amount of a RPL or RPS appears to impair 60S or 40S ribosomal subunit assembly within the cell, creating ribosomal stress that leads to activation of p53. These RPs also appear to function through RPL5 and RPL11, since knocking either one down simultaneously leads to decreased levels of p53 rather than increased levels (Fumagalli et al., 2009; Sun et al., 2010). RPL5 and RPL11 have also been shown to cooperate with each other for their function (Horn and Vousden, 2008), and one recent report suggested RPL11 and RPL5 may be especially important in transducing ribosomal stress signals to p53, since they accumulate in ribosomal-free fractions after ActD treatment, while several other RPs are rapidly degraded (Bursac et al., 2012).

Thus, a model was hypothesized where RPs can be classified as “detector” or “effector” regulators of p53 (Daftuar et al., 2010; Llanos and Serrano, 2010; Zhou et al., 2012). Effector RPs function as tumor suppressors

because they bind to Mdm2, upregulate p53 when overexpressed by inhibiting Mdm2-mediated ubiquitination, and are necessary for a full p53 response to ribosomal stress. Detector RPs do not bind to Mdm2, have no effect on p53 levels when overexpressed, but cause p53 levels to rise in an RPL11-dependent manner when knocked down by siRNA. Thus, detector RPs can be seen as indirect regulators of p53, and based upon this model, RPS12 may tentatively be classified as a detector RP, since a recent publication shows it fails to co-immunoprecipitate with Mdm2 and fails to inhibit p53 degradation in cells (Sun et al., 2011).

Additional roles for the RP-Mdm2-p53 pathway

Several lines of evidence suggest that the interaction between effector RPs and Mdm2 has relevance *in vivo*. Besides being essential for responding to ribosomal stress, several RPs have been shown to play a role in the p53 response to DNA damage. One report found that RPs move from the nucleolus to the nucleoplasm in a p53-dependent manner following etoposide treatment (Boisvert and Lamond, 2010), and RPS25 and RPL27L have been shown to be necessary for p53 levels to be fully induced (Xiong et al., 2011; Zhang et al., 2013). Similarly, ionizing radiation normally strongly induces p53, but when RPL26 or RPL27L is ablated by siRNA, p53 induction is attenuated (Sun et al., 2010; Xiong et al., 2011). Ablation of RPL11 or RPS7 by siRNA decreases p53 and Mdm2 stabilization after a wide variety of DNA damage agents such as

doxorubicin, daunorubicin, neocarzinostatin, and hydroxyurea (Zhu et al., 2009). Finally, if siRPS26 cells are treated with doxorubicin, they exhibit less acetyl-p53 and less transactivation of target genes such as Mdm2 and p21, although levels of total p53 and phospho-S15 p53 are maintained (Cui et al., 2013).

RPs have also been shown to be important in the response to other forms of stress, both p53-dependent and Mdm2-dependent. Knockdown of RPS3 by siRNA leads to an attenuation of p53 upregulation in response to hydrogen peroxide-induced oxidative stress (Yadavilli et al., 2009). And RPS7 was recently shown to inhibit Mdm2-mediated ubiquitination and degradation of Gadd45a, and be necessary for induction of cell death mediated by the carcinogen arsenite (Gao et al., 2013).

Furthermore, several studies demonstrate the importance of intact RP-Mdm2-p53 pathways in humans, fish, and mice. Human diseases caused by mutations to RP genes (such as Diamond-Blackfan anemia and 5q- syndrome) are characterized by a predisposition to cancer, although the p53 status in patients is unclear. But deletion of one allele of 17 different RPs in zebrafish is tumorigenic, and this was recently linked to lower levels of (wildtype) p53 protein in the tumors (MacInnes et al., 2008). Finally, only a few tumor-derived missense mutations have been identified in Mdm2, and at least two of them (C305F and C308Y) show impaired binding to RPL5 and RPL11. These mutations maintain their ability to interact with Arf and suppress p53 transactivation, but cannot degrade p53 as efficiently as wildtype Mdm2 (Lindstrom et al., 2007). Most

significantly, a Mdm2-C305F mouse has significantly accelerated tumor development in an Eμ-Myc model, and cells from the mouse fail to activate p53 in response to ActD, 5FU, or MPA -mediated ribosomal stress (Macias et al., 2010). Therefore, interactions between RPs and Mdm2 play important roles in tumor suppression.

NOVEL RIBOSOMAL RPOTEINS THAT MAY INTERACT WITH MDM2

A previous lab member, Dr. Xavier Jacq, performed an extensive yeast two-hybrid (Y2H) screen (Zhu et al., 2009) and identified over 500 potential Mdm2 interacting proteins. 11 of these fell into the ribosomal protein (RP) family (Figure 1.7), and 5 of them (RPL11, RPL26, RPS7, RPS26, and RPS27A) have been published as *bona fide* interactors with Mdm2 and negative regulators of Mdm2-mediated ubiquitination of p53 (Chen et al., 2007; Cui et al., 2013; Lohrum et al., 2003; Sun et al., 2011; Takagi et al., 2005; Zhang et al., 2003). The rest of this thesis describes 4 of the remaining RPs identified by the Y2H screen, namely RPL36A/RPL36AL, RPL37, RPS15, and RPS20.

REFERENCES

Abbas, T., and Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. Nature reviews. Cancer *9*, 400-414.

Alt, J.R., Bouska, A., Fernandez, M.R., Cerny, R.L., Xiao, H., and Eischen, C.M. (2005). Mdm2 binds to Nbs1 at sites of DNA damage and regulates double strand break repair. J Biol Chem *280*, 18771-18781.

Amsterdam, A., Sadler, K.C., Lai, K., Farrington, S., Bronson, R.T., Lees, J.A., and Hopkins, N. (2004). Many ribosomal protein genes are cancer genes in zebrafish. PLoS biology *2*, E139.

Anderson, S.J., Lauritsen, J.P., Hartman, M.G., Foushee, A.M., Lefebvre, J.M., Shinton, S.A., Gerhardt, B., Hardy, R.R., Oravecz, T., and Wiest, D.L. (2007). Ablation of ribosomal protein L22 selectively impairs alphabeta T cell development by activation of a p53-dependent checkpoint. Immunity *26*, 759-772.

Apicelli, A.J., Maggi, L.B., Jr., Hirbe, A.C., Miceli, A.P., Olanich, M.E., Schulte-Winkeler, C.L., Saporita, A.J., Kuchenreuther, M., Sanchez, J., Weilbaecher, K., *et al.* (2008). A non-tumor suppressor role for basal p19ARF in maintaining nucleolar structure and function. Molecular and cellular biology *28*, 1068-1080.

Badhai, J., Frojmark, A.S., E, J.D., Schuster, J., and Dahl, N. (2009). Ribosomal protein S19 and S24 insufficiency cause distinct cell cycle defects in Diamond-Blackfan anemia. Biochimica et biophysica acta *1792*, 1036-1042.

Badis, G., Saveanu, C., Fromont-Racine, M., and Jacquier, A. (2004). Targeted mRNA degradation by deadenylation-independent decapping. Molecular cell *15*, 5-15.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., *et al.* (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science *244*, 217-221.

Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science *289*, 905-920.

Baptiste, N., and Prives, C. (2004). p53 in the cytoplasm: a question of overkill? Cell *116*, 487-489.

Barkic, M., Crnomarkovic, S., Grabusic, K., Bogetic, I., Panic, L., Tamarut, S., Cokaric, M., Jeric, I., Vidak, S., and Volarevic, S. (2009). The p53 tumor suppressor causes congenital malformations in Rpl24-deficient mice and promotes their survival. Molecular and cellular biology *29*, 2489-2504.

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D.*, et al.* (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell *86*, 159-171.

Baroni, E., Viscardi, V., Cartagena-Lirola, H., Lucchini, G., and Longhese, M.P. (2004). The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation. Molecular and cellular biology *24*, 4151-4165.

Beckerman, R., and Prives, C. (2010). Transcriptional regulation by p53. Cold Spring Harb Perspect *2*, a000935.

Ben-Shem, A., Garreau de Loubresse, N., Melnikov, S., Jenner, L., Yusupova, G., and Yusupov, M. (2011). The structure of the eukaryotic ribosome at 3.0 A resolution. Science *334*, 1524-1529.

Bensaad, K., Tsuruta, A., Selak, M.A., Vidal, M.N., Nakano, K., Bartrons, R., Gottlieb, E., and Vousden, K.H. (2006). TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell *126*, 107-120.

Bertwistle, D., Sugimoto, M., and Sherr, C.J. (2004). Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. Molecular and cellular biology *24*, 985-996.

Bochkareva, E., Kaustov, L., Ayed, A., Yi, G.S., Lu, Y., Pineda-Lucena, A., Liao, J.C., Okorokov, A.L., Milner, J., Arrowsmith, C.H.*, et al.* (2005). Single-stranded DNA mimicry in the p53 transactivation domain interaction with replication protein A. Proceedings of the National Academy of Sciences of the United States of America *102*, 15412-15417.

Boisvert, F.M., and Lamond, A.I. (2010). p53-Dependent subcellular proteome localization following DNA damage. Proteomics *10*, 4087-4097.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J., and Lamond, A.I. (2007). The multifunctional nucleolus. Nature reviews. Molecular cell biology *8*, 574-585.

Boulon, S., Westman, B.J., Hutten, S., Boisvert, F.M., and Lamond, A.I. (2010). The nucleolus under stress. Molecular cell *40*, 216-227.

Boyd, M.T., Vlatkovic, N., and Rubbi, C.P. (2011). The nucleolus directly regulates p53 export and degradation. The Journal of cell biology *194*, 689-703.

Brady, C.A., Jiang, D., Mello, S.S., Johnson, T.M., Jarvis, L.A., Kozak, M.M., Kenzelmann Broz, D., Basak, S., Park, E.J., McLaughlin, M.E.*, et al.* (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell *145*, 571-583.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nature reviews. Cancer *9*, 701-713.

Budde, A., and Grummt, I. (1999). p53 represses ribosomal gene transcription. Oncogene *18*, 1119-1124.

Burger, K., Muhl, B., Harasim, T., Rohrmoser, M., Malamoussi, A., Orban, M., Kellner, M., Gruber-Eber, A., Kremmer, E., Holzel, M.*, et al.* (2010). Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. The Journal of biological chemistry *285*, 12416-12425.

Burma, S., Chen, B.P., Murphy, M., Kurimasa, A., and Chen, D.J. (2001). ATM phosphorylates histone H2AX in response to DNA double-strand breaks. The Journal of biological chemistry *276*, 42462-42467.

Bursac, S., Brdovcak, M.C., Pfannkuchen, M., Orsolic, I., Golomb, L., Zhu, Y., Katz, C., Daftuar, L., Grabusic, K., Vukelic, I.*, et al.* (2012). Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. Proceedings of the National Academy of Sciences of the United States of America *109*, 20467-20472.

Candeias, M.M., Malbert-Colas, L., Powell, D.J., Daskalogianni, C., Maslon, M.M., Naski, N., Bourougaa, K., Calvo, F., and Fahraeus, R. (2008). P53 mRNA controls p53 activity by managing Mdm2 functions. Nature cell biology *10*, 1098-1105.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Carter, S., and Vousden, K.H. (2009). Modifications of p53: competing for the lysines. Current opinion in genetics & development *19*, 18-24.

Carvajal, L.A., and Manfredi, J.J. (2013). Another fork in the road-life or death decisions by the tumour suppressor p53. EMBO reports *14*, 414-421.

Catic, A., and Ploegh, H.L. (2005). Ubiquitin--conserved protein or selfish gene? Trends in biochemical sciences *30*, 600-604.

Cavdar Koc, E., Burkhart, W., Blackburn, K., Moseley, A., and Spremulli, L.L. (2001). The small subunit of the mammalian mitochondrial ribosome. Identification of the full complement of ribosomal proteins present. The Journal of biological chemistry *276*, 19363-19374.

Cayrol, C., Knibiehler, M., and Ducommun, B. (1998). p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. Oncogene *16*, 311-320.

Challagundla, K.B., Sun, X.X., Zhang, X., DeVine, T., Zhang, Q., Sears, R.C., and Dai, M.S. (2011). Ribosomal protein L11 recruits miR-24/miRISC to repress c-Myc expression in response to ribosomal stress. Molecular and cellular biology *31*, 4007-4021.

Chehab, N.H., Malikzay, A., Appel, M., and Halazonetis, T.D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes & development *14*, 278-288.

Chen, D., and Huang, S. (2001). Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. The Journal of cell biology *153*, 169-176.

Chen, D., Zhang, Z., Li, M., Wang, W., Li, Y., Rayburn, E.R., Hill, D.L., Wang, H., and Zhang, R. (2007). Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene *26*, 5029-5037.

Chen, J. (2012). The Roles of MDM2 and MDMX Phosphorylation in Stress Signaling to p53. Genes & cancer *3*, 274-282.

Chen, J., Guo, K., and Kastan, M.B. (2012). Interactions of nucleolin and ribosomal protein L26 (RPL26) in translational control of human p53 mRNA. The Journal of biological chemistry *287*, 16467-16476.

Chen, J., and Kastan, M.B. (2010). 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes & development *24*, 2146-2156.

Chen, J., Marechal, V., and Levine, A.J. (1993). Mapping of the p53 and mdm-2 interaction domains. Molecular and cellular biology *13*, 4107-4114.

Chen, L., Gilkes, D.M., Pan, Y., Lane, W.S., and Chen, J. (2005). ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. The EMBO journal *24*, 3411-3422.

Chen, Y., Dey, R., and Chen, L. (2010). Crystal structure of the p53 core domain bound to a full consensus site as a self-assembled tetramer. Structure *18*, 246-256.

Chiocchetti, A., Zhou, J., Zhu, H., Karl, T., Haubenreisser, O., Rinnerthaler, M., Heeren, G., Oender, K., Bauer, J., Hintner, H.*, et al.* (2007). Ribosomal proteins Rpl10 and Rps6 are potent regulators of yeast replicative life span. Experimental gerontology *42*, 275-286.

Chipuk, J.E., and Green, D.R. (2006). Dissecting p53-dependent apoptosis. Cell death and differentiation *13*, 994-1002.

Chipuk, J.E., and Green, D.R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends in cell biology *18*, 157-164.

Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M., and Green, D.R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science *303*, 1010-1014.

Cho, Y., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346-355.

Chun, H.H., and Gatti, R.A. (2004). Ataxia-telangiectasia, an evolving phenotype. DNA repair *3*, 1187-1196.

Cicala, C., Pompetti, F., and Carbone, M. (1993). SV40 induces mesotheliomas in hamsters. The American journal of pathology *142*, 1524-1533.

Collot-Teixeira, S., Bass, J., Denis, F., and Ranger-Rogez, S. (2004). Human tumor suppressor p53 and DNA viruses. Reviews in medical virology *14*, 301-319.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121-134.

Cui, D., Li, L., Lou, H., Sun, H., Ngai, S.M., Shao, G., and Tang, J. (2013). The ribosomal protein S26 regulates p53 activity in response to DNA damage. Oncogene.

Daftuar, L., Zhu, Y., and Prives, C. (2010). Ribosomal protein L37 and the p53 network. Cell cycle *9*, 4259.

Dai, M.S., Arnold, H., Sun, X.X., Sears, R., and Lu, H. (2007). Inhibition of c-Myc activity by ribosomal protein L11. The EMBO journal *26*, 3332-3345.

Dai, M.S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. The Journal of biological chemistry *279*, 44475-44482.

Dai, M.S., Shi, D., Jin, Y., Sun, X.X., Zhang, Y., Grossman, S.R., and Lu, H. (2006). Regulation of the MDM2-p53 pathway by ribosomal protein L11 involves a post-ubiquitination mechanism. The Journal of biological chemistry *281*, 24304-24313.

Dai, M.S., Sun, X.X., and Lu, H. (2010). Ribosomal protein L11 associates with c-Myc at 5 S rRNA and tRNA genes and regulates their expression. The Journal of biological chemistry *285*, 12587-12594.

Dai, M.S., Zeng, S.X., Jin, Y., Sun, X.X., David, L., and Lu, H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Molecular and cellular biology *24*, 7654-7668.

Dawson, R., Muller, L., Dehner, A., Klein, C., Kessler, H., and Buchner, J. (2003). The N-terminal domain of p53 is natively unfolded. Journal of molecular biology *332*, 1131-1141.

De Bortoli, M., Castellino, R.C., Lu, X.Y., Deyo, J., Sturla, L.M., Adesina, A.M., Perlaky, L., Pomeroy, S.L., Lau, C.C., Man, T.K.*, et al.* (2006). Medulloblastoma outcome is adversely associated with overexpression of EEF1D, RPL30, and RPS20 on the long arm of chromosome 8. BMC cancer *6*, 223.

De Clercq, S., Gembarska, A., Denecker, G., Maetens, M., Naessens, M., Haigh, K., Haigh, J.J., and Marine, J.C. (2010). Widespread overexpression of epitope-tagged Mdm4 does not accelerate tumor formation in vivo. Molecular and cellular biology *30*, 5394-5405.

Degenhardt, R.F., and Bonham-Smith, P.C. (2008). Arabidopsis ribosomal proteins RPL23aA and RPL23aB are differentially targeted to the nucleolus and are disparately required for normal development. Plant physiology *147*, 128-142.

DeLeo, A.B., Jay, G., Appella, E., Dubois, G.C., Law, L.W., and Old, L.J. (1979). Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proceedings of the National Academy of Sciences of the United States of America *76*, 2420-2424.

Derenzini, M., Montanaro, L., and Trere, D. (2009). What the nucleolus says to a tumour pathologist. Histopathology *54*, 753-762.

Dever, T.E., and Green, R. (2012). The elongation, termination, and recycling phases of translation in eukaryotes. Cold Spring Harbor perspectives in biology *4*, a013706.

Dieci, G., Duimio, L., Coda-Zabetta, F., Sprague, K.U., and Ottonello, S. (1993). A novel RNA polymerase III transcription factor fraction that is not required for template commitment. The Journal of biological chemistry *268*, 11199-11207.

Dieci, G., Ruotolo, R., Braglia, P., Carles, C., Carpentieri, A., Amoresano, A., and Ottonello, S. (2009). Positive modulation of RNA polymerase III transcription by ribosomal proteins. Biochemical and biophysical research communications *379*, 489-493.

Doherty, L., Sheen, M.R., Vlachos, A., Choesmel, V., O'Donohue, M.F., Clinton, C., Schneider, H.E., Sieff, C.A., Newburger, P.E., Ball, S.E.*, et al.* (2010). Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. American journal of human genetics *86*, 222-228.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Ebert, B.L., Pretz, J., Bosco, J., Chang, C.Y., Tamayo, P., Galili, N., Raza, A., Root, D.E., Attar, E., Ellis, S.R.*, et al.* (2008). Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature *451*, 335-339.

el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nature genetics *1*, 45-49.

Eliyahu, D., Raz, A., Gruss, P., Givol, D., and Oren, M. (1984). Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature *312*, 646-649.

Ellis, S.R., and Lipton, J.M. (2008). Diamond Blackfan anemia: a disorder of red blood cell development. Current topics in developmental biology *82*, 217-241.
Eng, F.J., and Warner, J.R. (1991). Structural basis for the regulation of splicing of a yeast messenger RNA. Cell *65*, 797-804.

Erster, S., Mihara, M., Kim, R.H., Petrenko, O., and Moll, U.M. (2004). In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Mol Cell Biol *24*, 6728-6741.

Fewell, S.W., and Woolford, J.L., Jr. (1999). Ribosomal protein S14 of Saccharomyces cerevisiae regulates its expression by binding to RPS14B pre-mRNA and to 18S rRNA. Molecular and cellular biology *19*, 826-834.

Finch, R.A., Donoviel, D.B., Potter, D., Shi, M., Fan, A., Freed, D.D., Wang, C.Y., Zambrowicz, B.P., Ramirez-Solis, R., Sands, A.T.*, et al.* (2002). mdmx is a negative regulator of p53 activity in vivo. Cancer research *62*, 3221-3225.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell *57*, 1083-1093.

Finley, D., Bartel, B., and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature *338*, 394-401.

Fiscella, M., Zhang, H., Fan, S., Sakaguchi, K., Shen, S., Mercer, W.E., Vande Woude, G.F., O'Connor, P.M., and Appella, E. (1997). Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. Proceedings of the National Academy of Sciences of the United States of America *94*, 6048-6053.

Francoz, S., Froment, P., Bogaerts, S., De Clercq, S., Maetens, M., Doumont, G., Bellefroid, E., and Marine, J.C. (2006). Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. Proceedings of the National Academy of Sciences of the United States of America *103*, 3232-3237.

Fumagalli, S., Di Cara, A., Neb-Gulati, A., Natt, F., Schwemberger, S., Hall, J., Babcock, G.F., Bernardi, R., Pandolfi, P.P., and Thomas, G. (2009). Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an

rpL11-translation-dependent mechanism of p53 induction. Nature cell biology *11*, 501-508.

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Molecular and cellular biology *12*, 2866-2871.

Gajjar, M., Candeias, M.M., Malbert-Colas, L., Mazars, A., Fujita, J., Olivares-Illana, V., and Fahraeus, R. (2012). The p53 mRNA-Mdm2 interaction controls Mdm2 nuclear trafficking and is required for p53 activation following DNA damage. Cancer cell *21*, 25-35.

Gao, M., Li, X., Dong, W., Jin, R., Ma, H., Yang, P., Hu, M., Li, Y., Hao, Y., Yuan, S.*, et al.* (2013). Ribosomal protein S7 regulates arsenite-induced GADD45alpha expression by attenuating MDM2-mediated GADD45alpha ubiquitination and degradation. Nucleic acids research *41*, 5210-5222.

Garcia, D., Warr, M.R., Martins, C.P., Brown Swigart, L., Passeque, E., and Evan, G.I. (2011). Validation of MdmX as a therapeutic target for reactivating p53 in tumors. Genes Dev *25*, 1746-1757.

Garcia-Cao, I., Garcia-Cao, M., Martin-Caballero, J., Criado, L.M., Klatt, P., Flores, J.M., Weill, J.C., Blasco, M.A., and Serrano, M. (2002). "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. The EMBO journal *21*, 6225-6235.

Gazda, H.T., Sheen, M.R., Vlachos, A., Choesmel, V., O'Donohue, M.F., Schneider, H., Darras, N., Hasman, C., Sieff, C.A., Newburger, P.E.*, et al.* (2008). Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. American journal of human genetics *83*, 769-780.

Ghoshal, K., and Jacob, S.T. (1994). Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil. Cancer research *54*, 632-636.

Gilkes, D.M., Chen, L., and Chen, J. (2006). MDMX regulation of p53 response to ribosomal stress. The EMBO journal *25*, 5614-5625.

Giono, L.E., and Manfredi, J.J. (2007). Mdm2 is required for inhibition of Cdk2 activity by p21, thereby contributing to p53-dependent cell cycle arrest. Molecular and cellular biology *27*, 4166-4178.

Grossman, S.R., Deato, M.E., Brignone, C., Chan, H.M., Kung, A.L., Tagami, H., Nakatani, Y., and Livingston, D.M. (2003). Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science *300*, 342-344.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Hinds, P., Finlay, C., and Levine, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. Journal of virology *63*, 739-746.

Hirao, A., Kong, Y.Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S.J., and Mak, T.W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science *287*, 1824-1827.

Ho, W.C., Fitzgerald, M.X., and Marmorstein, R. (2006). Structure of the p53 core domain dimer bound to DNA. The Journal of biological chemistry *281*, 20494-20502.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. Annual review of genetics *30*, 405-439.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS letters *420*, 25-27.

Horn, H., and Vousden, K. (2008). Cooperation between the ribosomal proteins L5 and L11 in the p53 pathway. Oncogene *27*, 5774-5784.

Houtgraaf, J.H., Versmissen, J., and van der Giessen, W.J. (2006). A concise review of DNA damage checkpoints and repair in mammalian cells. Cardiovascular revascularization medicine : including molecular interventions *7*, 165-172.

Hu, W., Feng, Z., Teresky, A.K., and Levine, A.J. (2007). p53 regulates maternal reproduction through LIF. Nature *450*, 721-724.

Imafuku, I., Masaki, T., Waragai, M., Takeuchi, S., Kawabata, M., Hirai, S., Ohno, S., Nee, L.E., Lippa, C.F., Kanazawa, I.*, et al.* (1999). Presenilin 1 suppresses the function of c-Jun homodimers via interaction with QM/Jif-1. The Journal of cell biology *147*, 121-134.

Ito, A., Lai, C.H., Zhao, X., Saito, S., Hamilton, M.H., Appella, E., and Yao, T.P. (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. The EMBO journal *20*, 1331-1340.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature reviews. Molecular cell biology *11*, 113-127.

Jain, A.K., and Barton, M.C. (2010). Making sense of ubiquitin ligases that regulate p53. Cancer biology & therapy *10*, 665-672.

Jeffrey, P.D., Gorina, S., and Pavletich, N.P. (1995). Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. Science *267*, 1498-1502.

Jenner, L., Melnikov, S., de Loubresse, N.G., Ben-Shem, A., Iskakova, M., Urzhumtsev, A., Meskauskas, A., Dinman, J., Yusupova, G., and Yusupov, M. (2012). Crystal structure of the 80S yeast ribosome. Current opinion in structural biology *22*, 759-767.

Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Molecular and cellular biology *24*, 7669-7680.

Jin, Y., Lee, H., Zeng, S.X., Dai, M.S., and Lu, H. (2003). MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. The EMBO journal *22*, 6365-6377.

Joerger, A.C., and Fersht, A.R. (2008). Structural biology of the tumor suppressor p53. Annual review of biochemistry *77*, 557-582.

Joerger, A.C., and Fersht, A.R. (2010). The tumor suppressor p53: from structures to drug discovery. Cold Spring Harbor perspectives in biology *2*, a000919.

Jones, S.N., Hancock, A.R., Vogel, H., Donehower, L.A., and Bradley, A. (1998). Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America *95*, 15608-15612.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Juven-Gershon, T., Shifman, O., Unger, T., Elkeles, A., Haupt, Y., and Oren, M. (1998). The Mdm2 oncoprotein interacts with the cell fate regulator Numb. Molecular and cellular biology *18*, 3974-3982.

Kawai, H., Wiederschain, D., Kitao, H., Stuart, J., Tsai, K.K., and Yuan, Z.M. (2003a). DNA damage-induced MDMX degradation is mediated by MDM2. The Journal of biological chemistry *278*, 45946-45953.

Kawai, H., Wiederschain, D., and Yuan, Z.M. (2003b). Critical contribution of the MDM2 acidic domain to p53 ubiquitination. Molecular and cellular biology *23*, 4939-4947.

Khosravi, R., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y., and Shkedy, D. (1999). Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. Proceedings of the National Academy of Sciences of the United States of America *96*, 14973-14977.

Kim, H.D., Kim, T.S., Joo, Y.J., Shin, H.S., Kim, S.H., Jang, C.Y., Lee, C.E., and Kim, J. (2010). RpS3 translation is repressed by interaction with its own mRNA. Journal of cellular biochemistry *110*, 294-303.

Kim, J., Chubatsu, L.S., Admon, A., Stahl, J., Fellous, R., and Linn, S. (1995). Implication of mammalian ribosomal protein S3 in the processing of DNA damage. The Journal of biological chemistry *270*, 13620-13629.

Kitayner, M., Rozenberg, H., Kessler, N., Rabinovich, D., Shaulov, L., Haran, T.E., and Shakked, Z. (2006). Structural basis of DNA recognition by p53 tetramers. Molecular cell *22*, 741-753.

Klauck, S.M., Felder, B., Kolb-Kokocinski, A., Schuster, C., Chiocchetti, A., Schupp, I., Wellenreuther, R., Schmotzer, G., Poustka, F., Breitenbach-Koller, L.*, et al.* (2006). Mutations in the ribosomal protein gene RPL10 suggest a novel modulating disease mechanism for autism. Molecular psychiatry *11*, 1073-1084.

Klein, C., Planker, E., Diercks, T., Kessler, H., Kunkele, K.P., Lang, K., Hansen, S., and Schwaiger, M. (2001). NMR spectroscopy reveals the solution dimerization interface of p53 core domains bound to their consensus DNA. The Journal of biological chemistry *276*, 49020-49027.

Koc, E.C., Burkhart, W., Blackburn, K., Moyer, M.B., Schlatzer, D.M., Moseley, A., and Spremulli, L.L. (2001). The large subunit of the mammalian mitochondrial ribosome. Analysis of the complement of ribosomal proteins present. The Journal of biological chemistry *276*, 43958-43969.

Kodama, M., Otsubo, C., Hirota, T., Yokota, J., Enari, M., and Taya, Y. (2010). Requirement of ATM for rapid p53 phosphorylation at Ser46 without Ser/Thr-Gln sequences. Molecular and cellular biology *30*, 1620-1633.

Komili, S., Farny, N.G., Roth, F.P., and Silver, P.A. (2007). Functional specificity among ribosomal proteins regulates gene expression. Cell *131*, 557-571.

Kortlever, R.M., and Bernards, R. (2006). Senescence, wound healing and cancer: the PAI-1 connection. Cell cycle *5*, 2697-2703.

Kress, M., May, E., Cassingena, R., and May, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. Journal of virology *31*, 472-483.

Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kulikov, R., Winter, M., and Blattner, C. (2006). Binding of p53 to the central domain of Mdm2 is regulated by phosphorylation. The Journal of biological chemistry *281*, 28575-28583.

Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J., and Pavletich, N.P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science *274*, 948-953.

Lai, M.D., and Xu, J. (2007). Ribosomal proteins and colorectal cancer. Current genomics *8*, 43-49.

Lam, Y.W., Lamond, A.I., Mann, M., and Andersen, J.S. (2007). Analysis of nucleolar protein dynamics reveals the nuclear degradation of ribosomal proteins. Current biology : CB *17*, 749-760.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Lane, D.P., Cheok, C.F., Brown, C., Madhumalar, A., Ghadessy, F.J., and Verma, C. (2010a). Mdm2 and p53 are highly conserved from placozoans to man. Cell cycle *9*, 540-547.

Lane, D.P., Cheok, C.F., Brown, C.J., Madhumalar, A., Ghadessy, F.J., and Verma, C. (2010b). The Mdm2 and p53 genes are conserved in the Arachnids. Cell cycle *9*, 748-754.

Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261-263.

Lane, D.P., Madhumalar, A., Lee, A.P., Tay, B.H., Verma, C., Brenner, S., and Venkatesh, B. (2011). Conservation of all three p53 family members and Mdm2 and Mdm4 in the cartilaginous fish. Cell cycle *10*, 4272-4279.

Lane, D.P., and Verma, C. (2012). Mdm2 in evolution. Genes & cancer *3*, 320-324.

Laptenko, O., and Prives, C. (2006). Transcriptional regulation by p53: one protein, many possibilities. Cell death differ *13*, 951-961.

Lavigueur, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., and Bernstein, A. (1989). High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Molecular and cellular biology *9*, 3982-3991.

Lavin, M.F. (2007). ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. Oncogene *26*, 7749-7758.

Lessard, F., Morin, F., Ivanchuk, S., Langlois, F., Stefanovsky, V., Rutka, J., and Moss, T. (2010). The ARF tumor suppressor controls ribosome biogenesis by regulating the RNA polymerase I transcription factor TTF-I. Molecular cell *38*, 539-550.

Levine, A.J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nature reviews. Cancer *9*, 749-758.

Li, C., Chen, L., and Chen, J. (2002). DNA damage induces MDMX nuclear translocation by p53-dependent and -independent mechanisms. Molecular and cellular biology *22*, 7562-7571.

Li, M., and Gu, W. (2011). A critical role for noncoding 5S rRNA in regulating Mdmx stability. Molecular Cell *43*, 1023-1032.

Lindstrom, M.S., Jin, A., Deisenroth, C., White Wolf, G., and Zhang, Y. (2007). Cancer-associated mutations in the MDM2 zinc finger domain disrupt ribosomal protein interaction and attenuate MDM2-induced p53 degradation. Molecular and cellular biology *27*, 1056-1068.

Lindstrom, M.S., and Nister, M. (2010). Silencing of ribosomal protein S9 elicits a multitude of cellular responses inhibiting the growth of cancer cells subsequent to p53 activation. PloS one *5*, e9578.

Linke, K., Mace, P.D., Smith, C.A., Vaux, D.L., Silke, J., and Day, C.L. (2008). Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is

required for their ubiquitylation in trans. Cell death and differentiation *15*, 841-848.

Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell *17*, 43-52.

Llanos, S., and Serrano, M. (2010). Depletion of ribosomal protein L37 occurs in response to DNA damage and activates p53 through the L11/MDM2 pathway. Cell cycle *9*, 4005-4012.

Lohrum, M.A., Ludwig, R.L., Kubbutat, M.H., Hanlon, M., and Vousden, K.H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer cell *3*, 577-587.

Lohrum, M.A., Woods, D.B., Ludwig, R.L., Balint, E., and Vousden, K.H. (2001). C-terminal ubiquitination of p53 contributes to nuclear export. Molecular and cellular biology *21*, 8521-8532.

Lu, W.J., Amatruda, J.F., and Abrams, J.M. (2009). p53 ancestry: gazing through an evolutionary lens. Nature reviews. Cancer *9*, 758-762.

Lu, X., Ma, O., Nguyen, T.A., Jones, S.N., Oren, M., and Donehower, L.A. (2007). The Wip1 Phosphatase acts as a gatekeeper in the p53-Mdm2 autoregulatory loop. Cancer cell *12*, 342-354.

Macias, E., Jin, A., Deisenroth, C., Bhat, K., Mao, H., Lindstrom, M.S., and Zhang, Y. (2010). An ARF-independent c-MYC-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 Interaction. Cancer cell *18*, 231-243.

MacInnes, A.W., Amsterdam, A., Whittaker, C.A., Hopkins, N., and Lees, J.A. (2008). Loss of p53 synthesis in zebrafish tumors with ribosomal protein gene mutations. Proceedings of the National Academy of Sciences of the United States of America *105*, 10408-10413.

Malecka, K.A., Ho, W.C., and Marmorstein, R. (2009). Crystal structure of a p53 core tetramer bound to DNA. Oncogene *28*, 325-333.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A.*, et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science *250*, 1233-1238.

Maltzman, W., and Czyzyk, L. (1984). UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Molecular and cellular biology *4*, 1689-1694.

Malygin, A.A., Parakhnevitch, N.M., Ivanov, A.V., Eperon, I.C., and Karpova, G.G. (2007). Human ribosomal protein S13 regulates expression of its own gene at the splicing step by a feedback mechanism. Nucleic acids research *35*, 6414-6423.

Mancini, F., Di Conza, G., Pellegrino, M., Rinaldo, C., Prodosmo, A., Giglio, S., D'Agnano, I., Florenzano, F., Felicioni, L., Buttitta, F.*, et al.* (2009). MDM4 (MDMX) localizes at the mitochondria and facilitates the p53-mediated intrinsic-apoptotic pathway. The EMBO journal *28*, 1926-1939.

Mandke, P., Wyatt, N., Fraser, J., Bates, B., Berberich, S.J., and Markey, M.P. (2012). MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame. PloS one *7*, e42034.

Manfredi, J.J. (2010). The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes & development *24*, 1580-1589.

Marechal, V., Elenbaas, B., Piette, J., Nicolas, J.C., and Levine, A.J. (1994). The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. Molecular and cellular biology *14*, 7414-7420.

Markey, M., and Berberich, S.J. (2008). Full-length hdmX transcripts decrease following genotoxic stress. Oncogene *27*, 6657-6666.

Martins, C.P., Brown-Swigart, L., and Evan, G.I. (2006). Modeling the therapeutic efficacy of p53 restoration in tumors. Cell *127*, 1323-1334.

Matoba, S., Kang, J.G., Patino, W.D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P.J., Bunz, F., and Hwang, P.M. (2006). p53 regulates mitochondrial respiration. Science *312*, 1650-1653.

Maya, R., Balass, M., Kim, S.T., Shkedy, D., Leal, J.F., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y.*, et al.* (2001). ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes & development *15*, 1067-1077.

Mayo, L.D., Turchi, J.J., and Berberich, S.J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. Cancer research *57*, 5013-5016.

Mazumder, B., Sampath, P., Seshadri, V., Maitra, R.K., DiCorleto, P.E., and Fox, P.L. (2003). Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. Cell *115*, 187-198.

McGowan, K.A., Li, J.Z., Park, C.Y., Beaudry, V., Tabor, H.K., Sabnis, A.J., Zhang, W., Fuchs, H., de Angelis, M.H., Myers, R.M.*, et al.* (2008). Ribosomal mutations cause p53-mediated dark skin and pleiotropic effects. Nature genetics *40*, 963-970.

Meek, D.W., and Anderson, C.W. (2009). Posttranslational modification of p53: cooperative integrators of function. Cold Spring Harbor perspectives in biology *1*, a000950.

Mekhail, K., Khacho, M., Carrigan, A., Hache, R.R., Gunaratnam, L., and Lee, S. (2005). Regulation of ubiquitin ligase dynamics by the nucleolus. The Journal of cell biology *170*, 733-744.

Melero, J.A., Stitt, D.T., Mangel, W.F., and Carroll, R.B. (1979). Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. Virology *93*, 466-480.

Meulmeester, E., Frenk, R., Stad, R., de Graaf, P., Marine, J.C., Vousden, K.H., and Jochemsen, A.G. (2003). Critical role for a central part of Mdm2 in the ubiquitylation of p53. Molecular and cellular biology *23*, 4929-4938.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. European journal of biochemistry / FEBS *267*, 6321-6330.

Mitra, K., Schaffitzel, C., Shaikh, T., Tama, F., Jenni, S., Brooks, C.L., 3rd, Ban, N., and Frank, J. (2005). Structure of the E. coli protein-conducting channel bound to a translating ribosome. Nature *438*, 318-324.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., and Reed, J.C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene *9*, 1799-1805.

Momand, J., Jung, D., Wilczynski, S., and Niland, J. (1998). The MDM2 gene amplification database. Nucleic acids research *26*, 3453-3459.

Momand, J., Villegas, A., and Belyi, V.A. (2011). The evolution of MDM2 family genes. Gene *486*, 23-30.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Montanaro, L., Trere, D., and Derenzini, M. (2008). Nucleolus, ribosomes, and cancer. The American journal of pathology *173*, 301-310.

Montes de Oca Luna R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-206.

Mukhopadhyay, R., Ray, P.S., Arif, A., Brady, A.K., Kinter, M., and Fox, P.L. (2008). DAPK-ZIPK-L13a axis constitutes a negative-feedback module regulating inflammatory gene expression. Molecular cell *32*, 371-382.

Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Molecular cell *7*, 683-694.

Naora, H., Takai, I., Adachi, M., and Naora, H. (1998). Altered cellular responses by varying expression of a ribosomal protein gene: sequential coordination of enhancement and suppression of ribosomal protein S3a gene expression induces apoptosis. The Journal of cell biology *141*, 741-753.

Nevins, J.R. (2001). The Rb/E2F pathway and cancer. Human molecular genetics *10*, 699-703.

Ni, J.Q., Liu, L.P., Hess, D., Rietdorf, J., and Sun, F.L. (2006). Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes & development *20*, 1959-1973.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P.*, et al.* (1989). Mutations in the p53 gene occur in diverse human tumour types. Nature *342*, 705-708.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Ofir-Rosenfeld, Y., Boggs, K., Michael, D., Kastan, M.B., and Oren, M. (2008). Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. Molecular cell *32*, 180-189.

Okorokov, A.L., Sherman, M.B., Plisson, C., Grinkevich, V., Sigmundsson, K., Selivanova, G., Milner, J., and Orlova, E.V. (2006). The structure of p53 tumour suppressor protein reveals the basis for its functional plasticity. The EMBO journal *25*, 5191-5200.

Oliner, J.D., Pietenpol, J.A., Thiagalingam, S., Gyuris, J., Kinzler, K.W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature *362*, 857-860.

Ou, Y.H., Chung, P.H., Sun, T.P., and Shieh, S.Y. (2005). p53 C-terminal phosphorylation by CHK1 and CHK2 participates in the regulation of DNA-damage-induced C-terminal acetylation. Molecular biology of the cell *16*, 1684-1695.

Panic, L., Tamarut, S., Sticker-Jantscheff, M., Barkic, M., Solter, D., Uzelac, M., Grabusic, K., and Volarevic, S. (2006). Ribosomal protein S6 gene haploinsufficiency is associated with activation of a p53-dependent checkpoint during gastrulation. Molecular and cellular biology *26*, 8880-8891.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D., and Rotter, V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature *312*, 649-651.

Parant, J.M., Reinke, V., Mims, B., and Lozano, G. (2001). Organization, expression, and localization of the murine mdmx gene and pseudogene. Gene *270*, 277-283.

Perry, R.P. (2007). Balanced production of ribosomal proteins. Gene *401*, 1-3.

Perry, R.P., and Kelley, D.E. (1970). Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. Journal of cellular physiology *76*, 127-139.

Poyurovsky, M.V., Katz, C., Laptenko, O., Beckerman, R., Lokshin, M., Ahn, J., Byeon, I.J., Gabizon, R., Mattia, M., Zupnick, A.*, et al.* (2010). The C terminus of p53 binds the N-terminal domain of MDM2. Nature structural & molecular biology *17*, 982-989.

Poyurovsky, M.V., Priest, C., Kentsis, A., Borden, K.L., Pan, Z.Q., Pavletich, N., and Prives, C. (2007). The Mdm2 RING domain C-terminus is required for supramolecular assembly and ubiquitin ligase activity. The EMBO journal *26*, 90-101.

Presutti, C., Ciafre, S.A., and Bozzoni, I. (1991). The ribosomal protein L2 in S. cerevisiae controls the level of accumulation of its own mRNA. The EMBO journal *10*, 2215-2221.

Ray, P.S., and Fox, P.L. (2007). A post-transcriptional pathway represses monocyte VEGF-A expression and angiogenic activity. The EMBO journal *26*, 3360-3372.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

RIngshausen, I., O'Shea, C.C., Finch, A.J., Swigart, L.B., and Evan, G.I. (2006). Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo. Cancer Cell *10*, 501-514.

Rippin, T.M., Freund, S.M., Veprintsev, D.B., and Fersht, A.R. (2002). Recognition of DNA by p53 core domain and location of intermolecular contacts of cooperative binding. Journal of molecular biology *319*, 351-358.

Robledo, S., Idol, R.A., Crimmins, D.L., Ladenson, J.H., Mason, P.J., and Bessler, M. (2008). The role of human ribosomal proteins in the maturation of rRNA and ribosome production. Rna *14*, 1918-1929.

Rubbi, C.P., and Milner, J. (2003). Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. The EMBO journal *22*, 6068-6077.

Ruggero, D., and Pandolfi, P.P. (2003). Does the ribosome translate cancer? Nature reviews. Cancer *3*, 179-192.

Rutkowski, R., Hofmann, K., and Gartner, A. (2010). Phylogeny and function of the invertebrate p53 superfamily. Cold Spring Harbor perspectives in biology *2*, a001131.

Saeboe-Larssen, S., Lyamouri, M., Merriam, J., Oksvold, M.P., and Lambertsson, A. (1998). Ribosomal protein insufficiency and the minute syndrome in Drosophila: a dose-response relationship. Genetics *148*, 1215-1224.

Sakaguchi, K., Herrera, J.E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C.W., and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes & development *12*, 2831-2841.

Schadt, E.E., Molony, C., Chudin, E., Hao, K., Yang, X., Lum, P.Y., Kasarskis, A., Zhang, B., Wang, S., Suver, C.*, et al.* (2008). Mapping the genetic architecture of gene expression in human liver. PLoS biology *6*, e107.

Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F.*, et al.* (2000). Structure of functionally activated small ribosomal subunit at 3.3 angstroms resolution. Cell *102*, 615-623.

Schroder, P.A., and Moore, M.J. (2005). Association of ribosomal proteins with nascent transcripts in S. cerevisiae. Rna *11*, 1521-1529.

Schumacher, B., Mondry, J., Thiel, P., Weyand, M., and Ottmann, C. (2010). Structure of the p53 C-terminus bound to 14-3-3: implications for stabilization of the p53 tetramer. FEBS letters *584*, 1443-1448.

Schuwirth, B.S., Borovinskaya, M.A., Hau, C.W., Zhang, W., Vila-Sanjurjo, A., Holton, J.M., and Cate, J.H. (2005). Structures of the bacterial ribosome at 3.5 A resolution. Science *310*, 827-834.

Sharp, D.A., Kratowicz, S.A., Sank, M.J., and George, D.L. (1999). Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. The Journal of biological chemistry *274*, 38189-38196.

Shetty, S., Shetty, P., Idell, S., Velusamy, T., Bhandary, Y.P., and Shetty, R.S. (2008). Regulation of plasminogen activator inhibitor-1 expression by tumor suppressor protein p53. The Journal of biological chemistry *283*, 19570-19580.

Shi, D., Pop, M.S., Kulikov, R., Love, I.M., Kung, A.L., and Grossman, S.R. (2009). CBP and p300 are cytoplasmic E4 polyubiquitin ligases for p53. Proceedings of the National Academy of Sciences of the United States of America *106*, 16275-16280.

Shibue, T., Takeda, K., Oda, E., Tanaka, H., Murasawa, H., Takaoka, A., Morishita, Y., Akira, S., Taniguchi, T., and Tanaka, N. (2003). Integral role of Noxa in p53-mediated apoptotic response. Genes & development *17*, 2233-2238.

Shieh, S.Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes & development *14*, 289-300.

Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-334.

Srivastava, S., Zou, Z.Q., Pirollo, K., Blattner, W., and Chang, E.H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature *348*, 747-749.

Stad, R., Ramos, Y.F., Little, N., Grivell, S., Attema, J., van Der Eb, A.J., and Jochemsen, A.G. (2000). Hdmx stabilizes Mdm2 and p53. The Journal of biological chemistry *275*, 28039-28044.

Strachan, G.D., Jordan-Sciutto, K.L., Rallapalli, R., Tuan, R.S., and Hall, D.J. (2003). The E2F-1 transcription factor is negatively regulated by its interaction with the MDMX protein. Journal of cellular biochemistry *88*, 557-568.

Sun, X.X., Dai, M.S., and Lu, H. (2008). Mycophenolic acid activation of p53 requires ribosomal proteins L5 and L11. The Journal of biological chemistry *283*, 12387-12392.

Sun, X.X., DeVine, T., Challagundla, K.B., and Dai, M.S. (2011). Interplay between ribosomal protein S27a and MDM2 protein in p53 activation in response to ribosomal stress. The Journal of biological chemistry *286*, 22730-22741.

Sun, X.X., Wang, Y.G., Xirodimas, D.P., and Dai, M.S. (2010). Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation. The Journal of biological chemistry *285*, 25812-25821.

Sundqvist, A., Liu, G., Mirsaliotis, A., and Xirodimas, D.P. (2009). Regulation of nucleolar signalling to p53 through NEDDylation of L11. EMBO reports *10*, 1132-1139.

Symington, L.S., and Gautier, J. (2011). Double-strand break end resection and repair pathway choice. Annual review of genetics *45*, 247-271.

Takagi, M., Absalon, M.J., McLure, K.G., and Kastan, M.B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell *123*, 49-63.

Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F., and Minna, J.D. (1989). p53: a frequent target for genetic abnormalities in lung cancer. Science *246*, 491-494.

Takekawa, M., Adachi, M., Nakahata, A., Nakayama, I., Itoh, F., Tsukuda, H., Taya, Y., and Imai, K. (2000). p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. The EMBO journal *19*, 6517-6526.

Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature *404*, 42-49.

Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. Cell *133*, 612-626.

Tanimura, S., Ohtsuka, S., Mitsui, K., Shirouzu, K., Yoshimura, A., and Ohtsubo, M. (1999). MDM2 interacts with MDMX through their RING finger domains. FEBS letters *447*, 5-9.

Tidow, H., Melero, R., Mylonas, E., Freund, S.M., Grossmann, J.G., Carazo, J.M., Svergun, D.I., Valle, M., and Fersht, A.R. (2007). Quaternary structures of tumor suppressor p53 and a specific p53 DNA complex. Proceedings of the National Academy of Sciences of the United States of America *104*, 12324-12329.

Tyner, S.D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C*., et al.* (2002). p53 mutant mice that display early ageing-associated phenotypes. Nature *415*, 45-53.

Uchida, C., Miwa, S., Kitagawa, K., Hattori, T., Isobe, T., Otani, S., Oda, T., Sugimura, H., Kamijo, T., Ookawa, K*., et al.* (2005). Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. The EMBO journal *24*, 160-169.

Uhrinova, S., Uhrin, D., Powers, H., Watt, K., Zheleva, D., Fischer, P., McInnes, C., and Barlow, P.N. (2005). Structure of free MDM2 N-terminal domain reveals conformational adjustments that accompany p53-binding. Journal of molecular biology *350*, 587-598.

Uldrijan, S., Pannekoek, W.J., and Vousden, K.H. (2007). An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. The EMBO journal *26*, 102-112.

Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. Nature *445*, 661-665.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., and Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. The New England journal of medicine *319*, 525-532.

Volarevic, S., Stewart, M.J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S.C., and Thomas, G. (2000). Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. Science *288*, 2045-2047.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Wade, M., Li, Y.C., and Wahl, G.M. (2013). MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nature reviews. Cancer *13*, 83-96.

Wade, M., and Wahl, G.M. (2009). Targeting Mdm2 and Mdmx in cancer therapy: better living through medicinal chemistry? Molecular cancer research : MCR *7*, 1-11.

Wade, M., Wang, Y.V., and Wahl, G.M. (2010). The p53 orchestra: Mdm2 and Mdmx set the tone. Trends in cell biology *20*, 299-309.

Wan, F., Anderson, D.E., Barnitz, R.A., Snow, A., Bidere, N., Zheng, L., Hegde, V., Lam, L.T., Staudt, L.M., Levens, D.*, et al.* (2007). Ribosomal protein S3: a KH domain subunit in NF-kappaB complexes that mediates selective gene regulation. Cell *131*, 927-939.

Wang, Y.V., Leblanc, M., Wade, M., Jochemsen, A.G., and Wahl, G.M. (2009). Increased radioresistance and accelerated B cell lymphomas in mice with Mdmx mutations that prevent modifications by DNA-damage-activated kinases. Cancer cell *16*, 33-43.

Wanzel, M., Russ, A.C., Kleine-Kohlbrecher, D., Colombo, E., Pelicci, P.G., and Eilers, M. (2008). A ribosomal protein L23-nucleophosmin circuit coordinates Mizl function with cell growth. Nature cell biology *10*, 1051-1061.

Warner, J.R. (1999). The economics of ribosome biosynthesis in yeast. Trends in biochemical sciences *24*, 437-440.

Warner, J.R., and McIntosh, K.B. (2009). How common are extraribosomal functions of ribosomal proteins? Molecular cell *34*, 3-11.

Watson, K.L., Konrad, K.D., Woods, D.F., and Bryant, P.J. (1992). Drosophila homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system. Proceedings of the National Academy of Sciences of the United States of America *89*, 11302-11306.

Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nature cell biology *1*, 20-26.

Wells, M., Tidow, H., Rutherford, T.J., Markwick, P., Jensen, M.R., Mylonas, E., Svergun, D.I., Blackledge, M., and Fersht, A.R. (2008). Structure of tumor suppressor p53 and its intrinsically disordered N-terminal transactivation domain. Proceedings of the National Academy of Sciences of the United States of America *105*, 5762-5767.

Wimberly, B.T., Brodersen, D.E., Clemons, W.M., Jr., Morgan-Warren, R.J., Carter, A.P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. (2000). Structure of the 30S ribosomal subunit. Nature *407*, 327-339.

Wool, I.G. (1996). Extraribosomal functions of ribosomal proteins. Trends in biochemical sciences *21*, 164-165.

Xiong, S., Pant, V., Suh, Y.A., Van Pelt, C.S., Wang, Y., Valentin-Vega, Y.A., Post, S.M., and Lozano, G. (2010). Spontaneous tumorigenesis in mice overexpressing the p53-negative regulator Mdm4. Cancer research *70*, 7148-7154.

Xiong, S., Van Pelt, C.S., Elizondo-Fraire, A.C., Liu, G., and Lozano, G. (2006). Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. Proceedings of the National Academy of Sciences of the United States of America *103*, 3226-3221.

Xiong, X., Zhao, Y., He, H., and Sun, Y. (2011). Ribosomal protein S27-like and S27 interplay with p53-MDM2 axis as a target, a substrate and a regulator. Oncogene *30*, 1798-1811.

Xirodimas, D.P., Chisholm, J., Desterro, J.M., Lane, D.P., and Hay, R.T. (2002). P14ARF promotes accumulation of SUMO-1 conjugated (H)Mdm2. FEBS letters *528*, 207-211.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656-660.

Yadavilli, S., Mayo, L.D., Higgins, M., Lain, S., Hegde, V., and Deutsch, W.A. (2009). Ribosomal protein S3: A multi-functional protein that interacts with both p53 and MDM2 through its KH domain. DNA repair *8*, 1215-1224.

Yamamoto, K., Wang, Y., Jiang, W., Liu, X., Dubois, R.L., Lin, C.S., Ludwig, T., Bakkenist, C.J., and Zha, S. (2012). Kinase-dead ATM protein causes genomic

instability and early embryonic lethality in mice. The Journal of cell biology *198*, 305-313.

Yu, G., Allen, M., Andreeva, A., Fersht, A., and Bycroft, M. (2006). Solution structure of the C4 zinc finger domain of HDM2. Protein Science *15*, 384-389.

Zhai, W., and Comai, L. (2000). Repression of RNA polymerase I transcription by the tumor suppressor p53. Molecular and cellular biology *20*, 5930-5938.

Zhang, X., Wang, W., Wang, H., Wang, M.H., Xu, W., and Zhang, R. (2013). Identification of ribosomal protein S25 (RPS25)-MDM2-p53 regulatory feedback loop. Oncogene *32*, 2782-2791.

Zhang, Y., and Lu, H. (2009). Signaling to p53: ribosomal proteins find their way. Cancer cell *16*, 369-377.

Zhang, Y., Wang, J., Yuan, Y., Zhang, W., Guan, W., Wu, Z., Jin, C., Chen, H., Zhang, L., Yang, X.*, et al.* (2010). Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26. Nucleic acids research *38*, 6544-6554.

Zhang, Y., Wolf, G.W., Bhat, K., Jin, A., Allio, T., Burkhart, W.A., and Xiong, Y. (2003). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Molecular and cellular biology *23*, 8902-8912.

Zhang, Y., Xiong, Y., and Yarbrough, W.G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725-734.

Zhou, X., Hao, Q., Liao, J., Zhang, Q., and Lu, H. (2013a). Ribosomal protein S14 unties the MDM2-p53 loop upon ribosomal stress. Oncogene *32*, 388-396.

Zhou, X., Hao, Q., Liao, J.M., Liao, P., and Lu, H. (2013b). Ribosomal protein S14 negatively regulates c-Myc activity. The Journal of biological chemistry.

Zhou, X., Liao, J.M., Liao, W.J., and Lu, H. (2012). Scission of the p53-MDM2 Loop by Ribosomal Proteins. Genes & cancer *3*, 298-310.

Zhu, Y., Poyurovsky, M.V., Li, Y., Biderman, L., Stahl, J., Jacq, X., and Prives, C. (2009). Ribosomal protein S7 is both a regulator and a substrate of MDM2. Molecular cell *35*, 316-326.

FIGURE LEGENDS

Figure 1.1. Schematic of p53. The N-terminus of p53 contains two transactivation domains (TAD1/2) that are responsible for regulating transcription of target genes. A proline-rich region (PRR) lies in between them and the central DNA binding domain, which recognizes and binds to specific sequences in target genes. At the C-terminus of p53 is an oligomerization domain (OD) and a C-terminal domain (CTD) that is modified both under basal conditions (ubiquitinated by Mdm2) and in response to stress (acetylated by p300). Figure is taken from (Joerger and Fersht, 2010).

Figure 1.2. Schematic of Mdm2 and MdmX. Mdm2 and MdmX are negative regulators of p53 that inhibit p53 function by binding to its transactivation domain. Mdm2 can also function as a RING-type E3 ubiquitin ligase and target p53 for degradation, while the RING domain of MdmX is inactive as an E3 ubiquitin ligase. Mdm2 uses its N-terminus to bind to the N-terminus of p53 and its C-terminal RING domain to ubiquitinate to C-terminus of p53, while the middle of Mdm2 contains a conserved acidic domain, Zinc finger, and nuclear localization and export signals. MdmX also binds to the N-terminus of p53 using its N-terminus, and the two Mdm proteins interact with each other using their RING domains. The percent identities of the various domains are indicated. Figure is adapted from (Wade et al., 2010).

Figure 1.3. Model of p53 signaling pathway. Under conditions of cellular stress, p53 becomes modified such that Mdm2 can no longer bind to it and degrade it. This leads to an accumulation of p53, which can function as a transcription factor within the nucleus. The genes that p53 can transactivate determine various cellular outcomes such as cell cycle arrest or apoptosis. p53 can also stimulate apoptosis in a transcription-independent way by binding to the pro-apoptotic proteins Bcl2 or BclX to activate permeabilization of outer mitochondrial membranes.

Figure 1.4. Conservation of selected ribosomal proteins across species. Ribosomes are highly conserved structures across all forms of life, with larger, more complex structures in eukaryotes and simpler structures in prokaryotes. Research across decades led to a profusion of names for the various ribosomal proteins (RPs) found in *E. coli*, *S. cerevisiae*, and *H. sapiens*. Recently, a consensus nomenclature was proposed, and is summarized here for various RPs that will be further described. Figure is adapted from (Jenner et al., 2012).

Figure 1.5. Ribosomal proteins that interact with Mdm2. 11 ribosomal proteins (RPs) have been shown to interact with Mdm2 and affect p53 levels when overexpressed. The four RPLs and 7 RPSs that have been published to do so are summarized here.

Figure 1.6. Model for upregulation of p53 by ribosomal proteins. Defects in ribosome transcription, processing, or assembly can lead to nucleolar stress that triggers cell cycle arrest. In 2009, 5 ribosomal proteins (RPL5, RPL11, RPL23, RPL26, and RPS7) were known to increase p53 levels by inhibiting Mdm2-mediated degradation or by stimulating transcription of message; more have since been uncovered. Figure is taken from (Zhang and Lu, 2009).

Figure 1.7. Candidate ribosomal proteins from Mdm2 yeast two-hybrid screen. 11 ribosomal protein (RP) genes were identified as “hits” in a yeast two-hybrid screen utilizing Mdm2 as the bait (performed by Dr. Xavier Jacq at Hybrigenics in 2004). 5 of these have been shown to interact with Mdm2 in human cells, while 6 of these candidates are novel. 5 of the novel RP genes were cloned from a human cDNA library into the mammalian expression vector pcDNA3-Myc and their ability to interact with Mdm2 was confirmed by co-immunoprecipitation assays in the indicated cell line(s). Their effect on p53 levels was also determined.

FIGURE 1.1

FIGURE 1.2

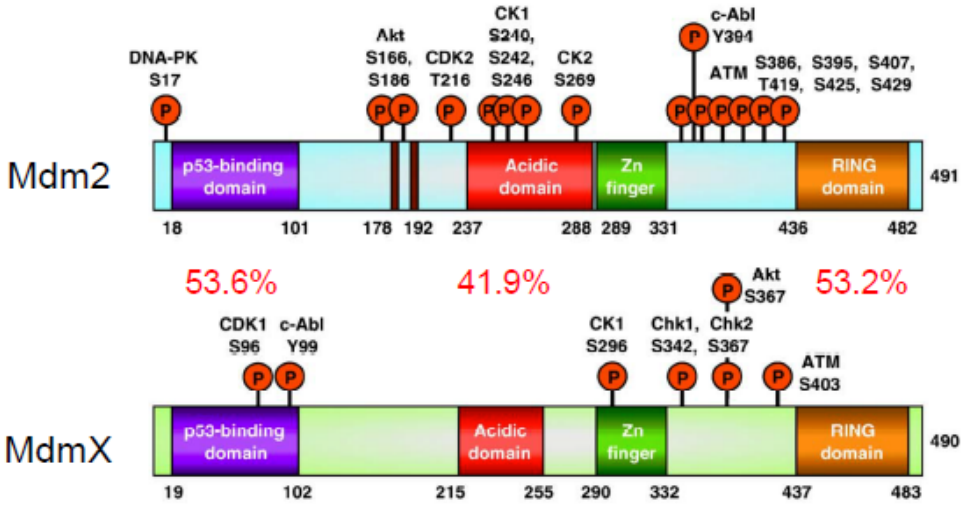

Mdm2
DNA-PK S17
Akt S166, S186
CDK2 T216
CK1 S240, S242, S246
CK2 S269
c-Abl Y394
ATM
S386, T419, S395, S425, S407, S429
p53-binding domain
Acidic domain
Zn finger
RING domain
491
18
101
178
192
237
288
289
331
436
482
53.6%
41.9%
53.2%
Akt S367
CDK1 S96
c-Abl Y99
CK1 S296
Chk1, S342,
Chk2 S367
ATM S403
MdmX
p53-binding domain
Acidic domain
Zn finger
RING domain
490
19
102
215
255
290
332
437
483

FIGURE 1.3

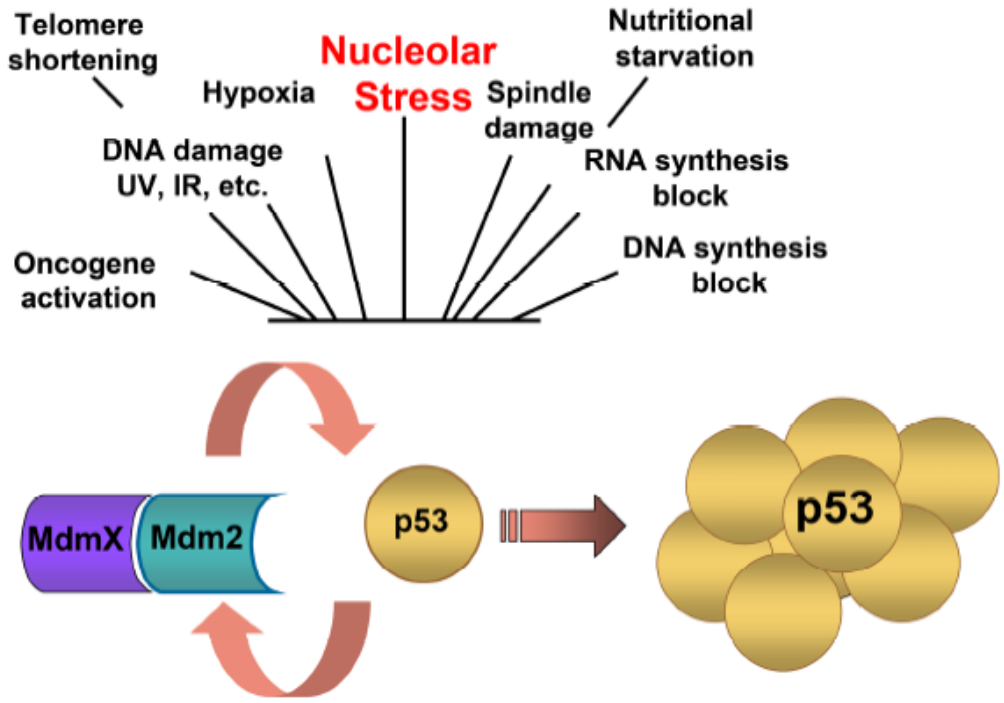

Telomere shortening
Nucleolar Stress
Nutritional starvation
Hypoxia
Spindle damage
DNA damage UV, IR, etc.
RNA synthesis block
Oncogene activation
DNA synthesis block
MdmX
Mdm2
p53
p53

FIGURE 1.4

Select ribosomal proteins and their conservation across species

	Human Name	Yeast Name	Bacteria Name
1	RPL5	RPL5	RPL18
2	RPL11	RPL11	RPL5
3	RPL12	RPL12	RPL11
4	RPL23	RPL23	RPL14
5	RPL26	RPL26	RPL24
6	RPL36A	RPL42	N/A
7	RPL37	RPL37	N/A
8	RPS3	RPS3	RPS3
9	RPS7	RPS7	N/A
10	RPS14	RPS14	RPS11
11	RPS15	RPS15	RPS19
12	RPS20	RPS20	RPS10
13	RPS25	RPS25	N/A
14	RPS26	RPS26	N/A
15	RPS27	RPS27	N/A
16	RPS27A	RPS31	N/A

FIGURE 1.5

Ribosomal proteins that interact with Mdm2, and their effects when overexpressed in cells

	RP	Effect on p53 levels?	Effect on Mdm2 levels?*	Reference
1	RPL5	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase	Dai and Lu, 2004
2	RPL11	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase (by inhibiting Mdm2 auto-ubiquitination)	Dai et al., 2006
3	RPL23	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase	Jin et al., 2004
4	RPL26	Increase (by inhibiting Mdm2-mediated ubiquitination and stimulating translation)	Increase (by inhibiting Mdm2 auto-ubiquitination)	Zhang et al., 2010
5	RPS7	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase (by inhibiting Mdm2 auto-ubiquitination)	Zhu et al., 2009
6	RPS14	Increase (by inhibiting Mdm2-mediated ubiquitination)	Not shown	Zhou et al., 2013
7	RPS25	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase (by inhibiting Mdm2 auto-ubiquitination)	Zhang et al., 2013
8	RPS26	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase (by inhibiting Mdm2 auto-ubiquitination)	Cui et al., 2013
9	RPS27	Increase (by inhibiting Mdm2-mediated ubiquitination)	Not shown	Xiong et al., 2011
10	RPS27A	Increase (by inhibiting Mdm2-mediated ubiquitination)	Increase	Sun et al., 2011
11	RPS27L	Increase (by inhibiting Mdm2-mediated ubiquitination)	Not shown	Xiong et al., 2011

*It is unclear if any of the RPs can also increase levels of Mdm2 by p53-mediated transcription.

FIGURE 1.6

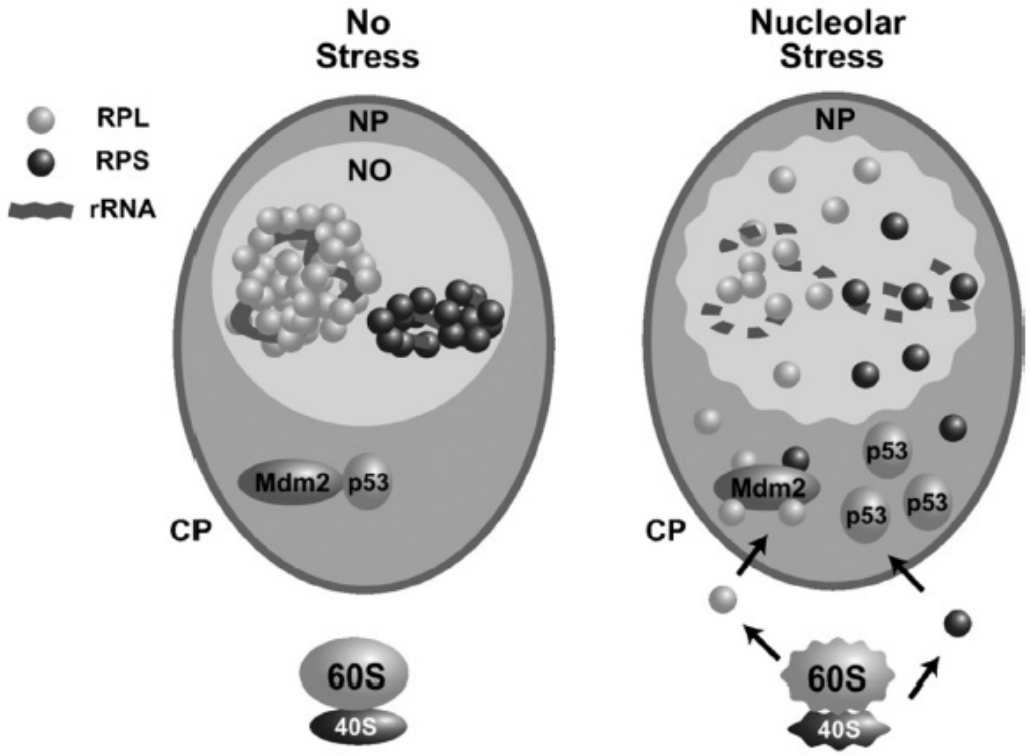

No Stress
Nucleolar Stress
RPL
RPS
rRNA
NP
NO
Mdm2
p53
CP
60S
40S
NP
p53
Mdm2
p53
p53
CP
60S
40S

FIGURE 1.7

Ribosomal proteins from yeast two-hybrid screen with Mdm2*

Gene	Physical interaction with Mdm2?	Effect on p53 levels when overexpressed?
1 RPL11 (published)	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
2 RPL36A	yes, co-IPs with Mdm2	decreases levels of p53
3 RPS7 (published)	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
4 RPS27A (published)	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
5 RPL12	yes, co-IPs with Mdm2	decreases levels of p53
6 RPL26 (published)	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
7 RPL37	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
8 RPS15	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
9 RPS20	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
10 RPS26 (published)	yes, co-IPs with Mdm2	increases levels of Mdm2, p53, and p21
11 MRPS5 (not cloned)	unknown	unknown

* Gray indicates previously published and results are from the literature

CHAPTER 2

Ribosomal proteins RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network

Lilyn Daftuar[1], Yan Zhu[1], Xavier Jacq[2,3] and Carol Prives[1,*]

[1] Department of Biological Sciences, Columbia University, New York, New York, United States of America

[2] Present address: MISSION Therapeutics Ltd, Cambridge, United Kingdom

[3] At the time of the study: Hybrigenics, Paris, France

* Corresponding Author: clp3@columbia.edu

ABSTRACT

Changes to the nucleolus, the site of ribosome production, have long been linked to cancer, and mutations in several ribosomal proteins (RPs) have been associated with an increased risk for cancer in human diseases. Relevantly, a number of RPs have been shown to bind to MDM2 and inhibit MDM2 E3 ligase activity, leading to p53 stabilization and cell cycle arrest, thus revealing a RP-Mdm2-p53 signaling pathway that is critical for ribosome biogenesis surveillance. Here, we have identified RPL37, RPS15, and RPS20 as RPs that can also bind Mdm2 and activate p53. We found that each of the aforementioned RPs, when ectopically expressed, can stabilize both co-expressed Flag-tagged Mdm2 and HA-tagged p53 in p53-null cells as well as endogenous p53 in a p53-containing cell line. For each RP, the mechanism of Mdm2 and p53 stabilization appears to be through inhibiting the E3 ubiquitin ligase activity of Mdm2. Interestingly, although they are each capable of inducing cell death and cell cycle arrest, these RPs differ in the p53 target genes that are regulated upon their respective introduction into cells. Furthermore, each RP can downregulate MdmX levels but in distinct ways. Thus, RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network but employ different mechanisms to do so.

INTRODUCTION

p53 is an important tumor suppressor in cells, and its loss or mutation has been implicated in at least half of all human cancers (Levine and Oren, 2009). Molecularly, p53 is a transcription factor that stimulates expression of numerous target genes in response to stress (Vousden and Prives, 2009). Levels of p53 are tightly regulated by Mdm2, a RING-type E3 ubiquitin ligase that binds to the N-terminal transactivation domain of p53 via sequences within its own N-terminal region. Mdm2 both inhibits p53 transactivation of its target genes and ubiquitinates lysines within the p53 C-terminus. Mdm2-mediated ubiquitination targets p53 for nuclear export and degradation by the proteasome (Toledo and Wahl, 2006). p53 activity is also regulated by MdmX, a homolog of Mdm2 that also contains a N-terminal p53-binding domain and a C-terminal RING domain (Shvarts et al., 1996). Just as with Mdm2, binding of the MdmX N-terminus to p53 inhibits its transactivation activity, but in the case of MdmX, its RING domain does not function to ubiquitinate p53. Rather, MdmX forms hetero-oligomers with Mdm2 in cells and likely directs Mdm2 RING activity towards p53 ubiquitination and away from Mdm2 auto-ubiquitination (Gu et al., 2002; Tollini and Zhang, 2012).

Upon some forms of cellular stress, MdmX is degraded, thus releasing p53 from inhibition (Biderman et al., 2012; Kawai et al., 2003), and Mdm2 and p53 are modified so that Mdm2 cannot bind to p53 and target it for degradation. Both mechanisms allow for a buildup of active p53 and arrest of the cell cycle or,

depending on the extent of the damage or cellular context, apoptosis. The accumulation of p53 also stimulates expression of Mdm2, thus completing an important negative feedback loop whereby p53 is eventually degraded once the stress has passed (Kruse and Gu, 2009). It is noteworthy that, unlike p53, Mdm2 and MdmX are only rarely mutated in human cancers; rather, they are sometimes amplified (Momand et al., 1998). The rare exceptions that have been identified for Mdm2 consist of a few missense mutations located within the central acidic region, which coincidentally is the same region that interacts with various ribosomal proteins (RPs).

While the nucleolus had already been linked to p53 by multiple lines of evidence (Derezini et al., 2009), the first report to directly link a ribosomal protein (RP) to p53 identified a physical interaction between Mdm2, p53, 5S rRNA, and RPL5 (Marechal et al., 1994). The significance of this interaction was unclear until it was published that RPL11 can also bind Mdm2, and overexpressing this RP allowed for the inhibition of the ubiquitination and degradation of p53 (Lohrum et al., 2003; Zhang et al., 2003). RPL11 was also shown to stimulate the Mdm2-mediated ubiquitination and degradation of MdmX (Gilkes et al., 2006).

The interaction between RPL11 and Mdm2 is not a unique phenomenon since it was then shown that p53 can be stabilized in an Mdm2-dependent manner by ectopic expression of RPL5, RPL23, RPS7, RPS14, RPS25, as well as RPS27, RPS27A, and RPS27L. These RPs all bind to the central region of Mdm2 and inhibit its E3 ubiquitin ligase activity, leading to the activation of p53

(Chen et al., 2007; Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Sun et al., 2011; Xiong et al., 2011; Zhang et al., 2013; Zhou et al., 2013; Zhu et al., 2007). Although knockdown of these RPs by siRNA have varying impacts on p53 protein levels in the absence of stress, they attenuate the induction of p53 when ribosomal stress is introduced to cells. For example, siRPL5 (Dai and Lu, 2004) and siRPL11 (Lohrum et al., 2003) reduce levels of p53 both basally and in response to stress, while siRPL23 and siRPS14 increase levels of basal p53 but attenuate the p53 response to ribosomal stress (Dai et al., 2004; Zhou et al., 2013). Furthermore, RPL5 and RPL11 were recently shown to accumulate in ribosomal-free fractions in response to actinomycin D (ActD)-induced ribosomal stress (Bursac et al., 2012). Interestingly, the few tumor-derived missense mutants of Mdm2 that have been identified impair binding to RPL5 and RPL11 while maintaining their interaction with p53 (Lindstrom et al., 2007). Furthermore, a mouse bearing one of these mutations (Mdm2-C305F) was shown to have significantly accelerated tumor development in an Eμ-Myc mouse model (Macias et al., 2010).

Another ribosomal protein, RPL26, was also shown to be a positive regulator of p53, but by more complex mechanisms. After irradiation of cells, RPL26 binds to both the 5’ UTR and 3’ UTR of p53 mRNA and stimulates its translation (Chen and Kastan, 2010; Takagi et al., 2005), while under non-stressed conditions, RPL26 is targeted for degradation by Mdm2 and is inhibited from interacting with p53 mRNA by Mdm2 (Ofir-Rosenfeld et al., 2008). More

recently, RPL26 was shown to stabilize p53 through inhibiting the ubiquitin ligase activity of Mdm2, similar to the RPs mentioned above (Zhang et al., 2010).

Interestingly, the role of RPs in regulating p53 signaling through Mdm2 interaction is not limited to ribosomal stress. It was shown that stress-induced p53 stabilization was attenuated by the various DNA damage agents after RPS7 or RPL11 ablation (Zhu et al., 2009). Also, another RP from the 40S small subunit, RPS3, was shown to interact with both p53 and Mdm2, and knockdown of RPS3 by siRNA led to an attenuation of p53 upregulation in response to oxidative stress (Yadavilli et al., 2009).

Our experiments identify three new RPs, RPL37, RPS15, and RPS20, that bind to and regulate Mdm2 and MdmX, and thereby p53. While they each appear to function similarly to many of the other RPs described above in their regulation of Mdm2, we noted interesting differences among them in the modes by which they interact with Mdm2, in their respective abilities to regulate p53 target gene expression, and how they regulate MdmX protein levels. Such differences might eventually provide a clue as to why so many different RPs appear to be involved in the Mdm2-p53-MdmX network.

MATERIALS AND METHODS

Plasmids and siRNA

Flag-Mdm2 (full length and deletion constructs), HA-MdmX, HA-p53, HA-ubiquitin, and GFP plasmids were described previously (Zhu et al., 2009). For Myc-RPs, total RNA was extracted from HEK293 cells (RNeasy Mini Kit, Qiagen) and a cDNA library was made by reverse-transcription (QuantiTect Reverse Transcription Kit, Qiagen). RPL37, RPS15, and RPS20 fragments were amplified from the cDNA library by PCR and cloned into the pcDNA3-Myc vector using the following primers: 5'-AAGGATCCAATGACGAAGGGAACGTCAT-3' and 5'-CCTCTAGATTAAGATGAACTGGATGCT-3' for RPL37 forward and reverse, respectively; 5'-AAGGATCCAATGGCAGAAGTAGAGCAGA-3' and 5'-CCGAATTCTTACTTGAGAGGGATGAAG-3' for RPS15 forward and reverse, respectively; and 5'-CCGGATCCAATGGCTTTTAAGGATACCG-3' and 5'-CCCTCGAGTTAAGCATCTGCAATGGTG-3' for RPS20 forward and reverse, respectively. DNA sequences were confirmed using the NCBI reference database. siRNA sequences (Table 2.T1) were obtained commercially (Qiagen); siRPs were pre-designed by the manufacturer while control siRNA (siRNA versus luciferase; siLuc) was previously described (Peart et al., 2010).

Cells culture and transfection

U2OS osteosarcoma, SJSA osteosarcoma, and H1299 lung carcinoma cell lines were described previously (Zhu et al., 2009). Cells were seeded in 35mM culture

plates prior to transfection, except where indicated. DNA transfections were performed for 24 hours with Lipofectamine 2000 (Invitrogen) or FuGENE6 (Promega) according to the manufacturer's instructions; siRNA transfections were performed for 72 hours with DharmFECT1 (Thermo Scientific) according to the manufacturer's instructions. All DNA transfections were balanced with pCDNA3-Myc to ensure equal amounts of total DNA were used, and all siRNA transfections were balanced with siLuciferase (Qiagen). Cells were harvested 24 hours after DNA transfection or 72 hours after siRNA transfection. Frozen cell pellets were stored at -80 °C until processed for RNA or protein analyses. In the case of cell cycle analyses, cells were processed immediately upon harvesting.

Antibodies and immunoblotting

Transfected cells were lysed with 100 µl Lysis Buffer (25 mM Tris-Hcl pH 7.5, 137 mM NaCl, 2.7 mM KCl, and 0.5% Igepal CA-630 supplemented with 50 nM PMSF and inhibitor cocktail containing 100uM Benzamidine, 300ug/uL Leupeptin, 100mg/mL Bacitracin, and 1mg/mL a_2-macroglobulin), and cell lysates were cleared by spinning at 4,000 rpm for 10 minutes. Protein concentrations were determined by Bradford assay, and equivalent amounts of each transfected and clarified cell lysate was loaded onto a polyacrylamide gel and separated using constant voltage. Proteins were transferred onto nitrocellulose membranes (Bio-Rad), blocked, and probed using the following antibodies: anti-actin (A2066, Sigma); anti-Flag (M2, Sigma); anti-GFP (B2,

Santa Cruz Biotechnology); anti-HA (16B12, Covance); anti-Mdm2 (a mixture of 3G5, 4B11, and 5B10 hybridomas); anti-MdmX (A300-287A, Bethyl Laboratories); anti-Myc (9E10, Santa Cruz Biotechnology or C3956, Sigma); anti-p21 (C19, Santa Cruz Biotechnology); anti-p53 (a mixture of 1801 and D01 hybridomas); anti-RPL37 (AP95656, Abgent); anti-RPS15 (AP6914a, Abgent); anti-RPS20 (ab74700, Abcam). Membranes were washed with PBS supplemented with 0.1% Tween 20 prior to the addition of secondary antibodies. In some cases, a goat anti-mouse or goat anti-rabbit conjugated to horseradish-peroxidase (Sigma) was used, and membranes were visualized using ECL (GE Healthcare). In other cases, fluorescent green goat anti-mouse (IRDye 800CW, LI-COR Biosciences) and fluorescent red donkey anti-rabbit (IRDye 680LT, LI-COR Biosciences) secondary antibodies were used in conjunction with the Odyssey Imaging System (LI-COR Biosciences).

Immunoprecipitations

H1299 cells were transfected with Myc-RPs, various Flag-Mdm2 constructs, or HA-MdmX as indicated. Equivalent amounts of each clarified cell lysate was subjected to immunoprecipitation with 1 μg of a monoclonal Myc antibody (9E10, Santa Cruz Biotechnology). For co-immunoprecipitation of endogenous proteins, confluent SJSA cells were lysed and cleared as described. SJSA cell lysates were pre-cleared with Protein G Sepharose beads (GE Healthcare) before immunoprecipitating with 1 μg of normal rabbit IgG (IB140, Sigma) or 1 μg of a

monoclonal Mdm2 antibody (N20, Santa Cruz Biotechnology). Pre-blocked Protein G Sepharose beads were added to cell lysates for 45 minutes, and unbound proteins were removed by washing with Lysis Buffer. Samples were resuspended in Lysis Buffer and Protein Sample Buffer prior to boiling at 95 °C for 10 minutes.

Ubiquitination assays

H1299 cells were transfected with HA-Ubiquitin, p53, Flag-Mdm2, or Myc-RPs as indicated. 18 hours after transfection, H1299 cells were treated with 25 μM MG132 (Calbiochem) for 6 hours. Equivalent amounts of clarified cell lysates were immunoprecipitated with 1 μg of anti-p53 (in the case of Mdm2-mediated ubiquitination of transfected p53) or 1.9 μg of anti-Flag (in the case of auto-ubiquitination of transfected Flag-Mdm2) followed by Western blot using anti-HA antibody to detect ubiquitinated p53 or Mdm2 species.

Cycloheximide assay

U2OS cells were transfected with empty vector (pcDNA3-Myc) or the indicated Myc-tagged RP as indicated. Approximately 23 hours after transfection, cells were treated with 100 μg/mL cycloheximide (Sigma) and harvested at the indicated time points. Cell lysates were subjected to immunoblotting with anti-p53 and anti-actin antibodies, and band intensities were quantified using Odyssey software (LI-COR Biosciences). After normalizing the p53 band intensities to

actin, the protein half-life of p53 was calculated using a one-phase exponential decay model (GraphPad Prism).

Quantitative RT-PCR

U2OS cells were transfected with Myc-RP as indicated. Total RNA was extracted from transfected cell pellets using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Expression of each gene was determined in triplicate using SYBR Green (Applied Biosystems) on a StepOnePlus Real-Time PCR machine (Applied Biosystems). Each sample was normalized using GAPDH primers, and relative gene expressions were determined using the ΔΔCt method. Primer sequences (Table 2.T2) were designed using Primer Express software (Applied Biosystems) and validated for efficiency and specificity prior to the start of experimentation.

Cell cycle analysis

U2OS cells were transfected with Myc-RP as indicated. Twenty-four hour after transfection, transfected cells were fixed and stained with propidium iodide (PI) as previously described (Peart et al., 2010) and analyzed using a FACS Calibur machine (BD Biosciences). Cell cycle distribution was determined using the ModFit LT program (Verity House Software), and sub-G1 content was determined using CellQuest software (BD Biosciences).

RESULTS

RPL37, RPS15, and RPS20 interact with Mdm2

A yeast two-hybrid screen using Mdm2 as bait (Zhu et al., 2009) identified several RPs as potential interactors with Mdm2. Among the candidate Mdm2-interacting RPs identified were RPL11 (Lohrum et al., 2003; Zhang et al., 2003), RPL26 (Takagi et al., 2005), RPS7 (Chen et al., 2007; Zhu et al., 2009), and RPS27A (Sun et al., 2011) which have been confirmed in published studies as *bona fide* interactors with Mdm2 and regulators of p53. Therefore, we sought to validate the additional RPs identified in the yeast two-hybrid screen by testing the interaction of RPL37, RPS15, and RPS20 with Mdm2 in mammalian cells. RPL37, RPS15, and RPS20 were cloned into a mammalian expression vector and transfected into H1299 lung carcinoma cells alongside Mdm2. When the transfected RPs were immunoprecipitated from the cell lysates, we found Mdm2 could associate with each of these RPs (Figures 2.1a-c). Significantly, the H1299 cell line is p53-null, indicating that the interactions of these three RPs with Mdm2 is independent of p53. The presence of p53 did not abrogate the interactions, however, as a similar result was observed with transfected RPs and Mdm2 in the p53-containing U2OS osteosarcoma cell line (Figure 2.S1). We also confirmed an interaction between the endogenously expressed RPs and Mdm2 in the SJSA osteosarcoma cell line that harbors wild-type p53 (Figure 2.1d).

We next sought to map the regions of Mdm2 that are responsible for interacting with these proteins. Other RPs (RPL5, RPL11, RPL23, RPS7, and

RPS14) have each been shown to bind to the central acidic region or central Zinc finger region of Mdm2 (Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003; Zhou et al., 2013; Zhu et al., 2009). Using a panel of Mdm2 deletion constructs, we found that RPL37, RPS15, and RPS20 can also bind to the central Zinc finger region of Mdm2, between amino acids 273-339 (Figure 2.1e). A Mdm2 variant lacking residues 340-437 bound better than full-length Mdm2 to each of the RPs, suggesting the region spanning amino acids 340-437 inhibits their interaction (Figure 2.S2). Interestingly as well, RPS15 and RPS20 but not RPL37 can also interact (albeit weakly) with the N-terminal 220 amino acids of Mdm2, where the p53-interacting domain lies, as well as the C-terminus of Mdm2, where the RING domain lies (Figure 2.S2). This suggests either that the tertiary structure of MDM2 augments its interaction with these two RPs or that they possess additional binding surfaces for Mdm2.

RPL37, RPS15, and RPS20 stabilize Mdm2 and p53 by inhibiting Mdm2 ubiquitin ligase activity

To determine the functional consequence of the physical interaction between RPL37, RPS15, and RPS20 and Mdm2, H1299 cells were transfected with a constant quantity of Flag-Mdm2 and increasing amounts of each Myc-RP. Levels of Flag-Mdm2 were increased by co-transfected RPs in a dose-dependent manner (Figure 2.2a). A similar stabilization of Flag-Mdm2 by these three RPs was seen when cells that were co-transfected Mdm2 and RPs were visualized by

immuofluoresent microscopy (Figure 2.S3). As Mdm2 may control its own degradation through its ubiquitin ligase activity, we introduced HA-tagged ubiquitin into H1299 cells and confirmed that each RP can inhibit Mdm2 auto-ubiquitination (Figures 2.2b-d).

We further examined whether the ability of these RPs to regulate Mdm2 levels had an impact on p53 levels. As shown in Figure 2.3a, when each RP was co-expressed in U2OS cells along with Flag-Mdm2 and HA-p53, they were able to inhibit Mdm2-mediated degradation of p53. More significantly, levels of endogenous Mdm2 and p53 in U2OS cells were elevated following expression of each RP in a dose-dependent manner (Figures 2.3b-d). Note as well that levels of p21 protein were increased along with p53.

Consistent with the above observations, cycloheximide chase assays revealed that the half-life of p53 was dramatically increased by the addition of each RP (Figures 2.4a-c). RPL37 was able to roughly double the half-life of p53, while RPS15 and RPS20 were able to extend the half-life of p53 by more than 4-fold. The underlying mechanism responsible for the ability of each RP to inhibit Mdm2-mediated degradation of p53 was obtained from experiments showing that that each RP inhibits Mdm2-mediated ubiquitination of p53 *in vivo* (Figures 2.4d-f). Together, these data suggest that ectopically expressed RPL37, RPS15, and RPS20 regulate Mdm2 and p53 levels by binding to Mdm2 and inhibiting its E3 ubiquitin ligase activity towards itself and p53.

RPL37, RPS15, and RPS20 increase cell death and cell cycle arrest

To examine the physiological consequence of the ectopic expression of RPL37, RPS15, or RPS20, we examined the cell cycle profiles of U2OS cells transfected with each of the RPs by FACS analysis. We found the overexpression of each of the three RPs was able to modestly, but significantly, increase the sub-G1 content of transfected U2OS cells in a dose-dependent manner, indicating that they can facilitate programmed cell death in this setting (Figure 2.5a). Surprisingly, RPL37 expression had only a negligible impact on the proportion of cells in G1 phase even though p21 protein levels were increased (Figure 2.3b). Instead, we found that RPL37 mediated a significant G2 arrest and a mild drop in S phase (Figures 2.5b-d). In the case of RPS15, a significant G2 arrest was seen with a mild drop in G1 phase, and the G2 arrest mediated by RPS20 correlated with modest drops in both G1 and S phases (Figures 2.5b-d). p21 has previously been shown to mediate G2 arrest in response to gamma irradiation (Bunz et al., 1998), but it remains possible that a different target (or targets) of p53 is regulated by the three RPs to cause cells to arrest in G2.

Stabilization of p53 by RPL37, RPS15, and RPS20 leads to upregulation of specific p53 targets

Upon treatment of cells with agents such as actinomycin D (ActD) or 5-fluorouracil (5-FU) that cause ribosomal stress, p53 becomes stabilized and can

activate its myriad downstream target genes. Since it is possible that one of the modes by which such ribosomal stress activates p53 is through freed RPs arising from nucleolar disruption, we sought to determine how the three RPs that we characterized in this study affect the transcriptional activity of p53. As a transcription factor, p53 increases expression of genes which can participate in cell cycle arrest (such as p21) or apoptosis (such as Bax, Noxa, and Puma) or both (such as miR-34a, a micro RNA target of p53 (Hermeking, 2007) that indirectly causes an increase in p21 and Puma expression by inhibiting Sirt1 (Yamakuchi et al., 2008)). p53 can also regulate metabolic flux through targets such as TIGAR (Bensaad et al., 2006) and can regulate itself through targets such as Mdm2 (Wade et al., 2013) and Ccng1 (Ohtsuka et al., 2004; Okamoto et al., 2002). As mentioned above, the ability of certain RPs to stabilize p53 with an ensuing cell outcome has been well documented. With few exceptions (Mahata et al., 2012; Morgado-Palacin et al., 2012), the impact of RPs on the ability of p53 to regulate its various target genes has not been examined. We therefore checked a few select but key p53 responsive genes for an *in vivo* response to ectopic expression of RPL37, RPS15, and RPS20.

Interestingly, not only did different p53 target genes vary in their response to ectopic RP expression, the 3 RPs differed among themselves in their ability to regulate expression of some of these genes. Consistent with our observation that RPL37, RPS15, and RPS20 could cause a dose-dependent increase in p21 protein levels (Figures 2.3b-3d), p21 mRNA accumulation was also increased

when each of the 3 RPs were expressed (Figure 2.6a). Expression of each RP also led to increased Puma mRNA levels (Figure 2.6b). In other cases, p53 target genes were induced by only a subset of the three RPs. Specifically, RPS15 and RPS20, but not RPL37, were able to increase mRNA levels of Mdm2 (Figure 2.6c) and miR-34a (Figure 2.6d) in a dose-dependent manner. Finally, expression of a third category of p53 targets (Ccng1, Bax, Noxa and Tigar) was not affected by these RPs to a significant degree (Figure 2.S4a-d).

Downregulation of MdmX protein levels by RPL37, RPS15, and RPS20

To further investigate possible differences in the *in vivo* functions of RPL37, RPS15, and RPS20, we asked whether they may play a role in the regulation of MdmX. RPL11 was previously shown to indirectly downregulate MdmX levels in a Mdm2-ubiquitination dependent manner (Gilkes et al., 2006), but the ability of other RPs to regulate MdmX has not been explored. When the RPs were immunoprecipitated from H1299 cell lysates transfected with individual Myc-tagged RPs and HA-tagged MdmX, RPS15 and RPS20 but not RPL37 co-immunoprecipitated with MdmX (Figures 2.7a-c). It is interesting that RPS15 and RPS20 were the RPs that exhibited weak interactions with the N-terminal and RING domains of Mdm2, while RPL37 did not interact with those regions of Mdm2 (Figure 2.1e, Figures 2.S2). The N-terminal domains of Mdm2 and MdmX share the highest sequence homology with each other (Shvarts et al., 1996), and

the RING domains of Mdm2 and MdmX are the sites of interaction between the two Mdm proteins (Linke et al., 2008; Tanimura et al., 1999).

Despite the absence of a physical interaction between RPL37 and MdmX proteins, ectopic expression of RPL37 was able to cause a dose-dependent drop in MdmX levels (Figure 2.7d), as were RPS15 and RPS20 (Figures 2.7e-f). As a possible explanation for the apparent contradiction between RPL37 regulating MdmX protein levels without binding to the protein, levels of MdmX mRNA were assayed. RPL37, but not RPS15 and RPS20, was able to cause a reduction in MdmX mRNA levels (Figure 2.7g), suggesting it has a different mechanism for regulating MdmX protein levels than the other RPs.

Knockdown of RPL37, RPS15, and RPS20 by siRNA increase levels of p53 and p21 but decrease levels of MdmX

As mentioned in the Introduction, siRNA-mediated depletion of some RPs that were shown to interact with Mdm2 is correlated with a decrease in p53 levels, while reduction of other RPs by siRNA was shown to perturb ribosomal biogenesis and lead to activation of p53. RPL37 was previously reported to fall in the latter category of RPs, as a siRNA targeting RPL37 was shown to activate p53 (Llanos and Serrano, 2010). Indeed, using a different siRNA sequence to deplete RPL37 also led to upregulation of p53 and p21 (Figure 2.8a). Similarly, siRNA mediated ablation of RPS15 and RPS20 led to upregulation of p53 and p21 (Figures 2.8b-c).

The effect of siRNA-mediated knockdown of RPs on MdmX other than siRPL11 (Gilkes et al., 2006) has not been widely reported. Here we found that siRPL37, siRPS15, and siRPS20 could also lead to a decrease in MdmX protein levels. Given the uniformity of this response, it is possible that, just like stressing cells with low doses of ActD leads to MdmX degradation, depleting RPs may introduce ribosomal stress that lead cells to decrease levels of MdmX protein.

DISCUSSION

The three ribosomal proteins that are the focus of this paper have each been previously implicated in activating p53 or suppressing cancer. Ablation of RPL37 by siRNA was shown to disrupt ribosomal biogenesis and upregulate p53 (Llanos and Serrano, 2010), and also lead to the upregulation of a variety of p53 targets (Morgado-Palacin et al., 2012). A case of Diamond-Blackfan anemia, a heritable human disorder characterized by a predisposition to cancer, was identified where RPS15 had been mutated (Gazda et al., 2008). In a genetic screen, RPS15 was identified as a haploinsufficient tumor suppressor in zebrafish (Amsterdam et al., 2004). Similarly, a mouse carrying a mutation in RPS20 was found to have activated p53 that leads to both anemia (due to an increase in apoptosis of erythrocytes) and darkened skin (due to an increase in the proliferation of melanocytes) (McGowan et al., 2008). Here we show that ablation of these three RPs by siRNA can lead to increased levels of p53, as can overexpression.

The experiments in this study have relied extensively on analysis of ectopically expressed ribosomal proteins. As such, they have both confirmed and extended observations made with other RPs, which, when similarly introduced into cells, lead to inhibition of Mdm2 activity and thereby stabilization of both p53 and Mdm2. While arguments that ectopically expressed proteins may be present at levels that are non-physiologically high are certainly valid in the case of many proteins, ribosomal proteins themselves are normally among the most abundant

proteins in the cell, and the amount that we are adding to the cellular pool is therefore unlikely to make a significant difference. Rather what we think we are accomplishing in our experiments is mimicking the situation that occurs upon ribosomal stress, which features nucleolar disruption and dispersal of free ribosomal proteins. Although some ribosomal proteins are rapidly degraded after some forms of ribosomal stress, others (RPL5 and RPL11) are stable (Bursac et al., 2012). To gain more insight into the likely roles of these proteins, it might be appropriate to further examine the consequences of depletion of these RPs on the p53 pathway in future studies. However, there are arguments that such approaches might not be that informative. In many cases, knockdown of RPs induces rather than suppresses activation of p53 due to the relationship between RPL11 and other RPs first noted by Thomas and colleagues (Fumagalli et al., 2009) and later by Dai and colleagues (Sun et al., 2010), and siRPL37, siRPS15, and siRPS20 may function similarly. The former found that disruption of 40S ribosome biogenesis mediated by siRPS6 causes arrest of the cell cycle in an RPL11-dependent manner, and the latter found that perturbation of 60S ribosome biogenesis mediated by siRPL29 or siRPL30 results in a similar outcome. A second argument is that siRNAs targeting even the same RP (e.g. RPS7) seem to provide different results in different reports (e.g. compare (Chen et al., 2007) and (Zhu et al., 2009) versus (Fumagalli et al., 2012) and (Bursac et al., 2012)). Therefore, we feel that the results in this paper provide new information about the relationship between RPs and p53 as discussed below.

Increasing numbers of RPs have been shown to contribute to p53 stress response. It was recently hypothesized that those RPs can be classified as "detector" RPs or "effector" RPs (Daftuar et al., 2010; Llanos and Serrano, 2010). Effector RPs, such as RPL11, can inhibit Mdm2-mediated ubiquitination and degradation of p53 when overexpressed, and most of those have also been shown to attenuate the response to stress when knocked down by siRNA. On the other hand, detector RPs, such as RPL7A, RPL24, RPL29, RPL30, RPL37, RPS6, RPS23, and RPS9 (Barkic et al., 2009; Fumagalli et al., 2009; Lindstrom and Nister, 2010; Llanos and Serrano, 2010; Sun et al., 2010), have an effect on p53 levels only when reduced by siRNA ─ they do not co-immunoprecipitate with Mdm2 and have no effect on p53 levels when they are overexpressed. These RPs appear to contribute to the p53 stress response by triggering an increase in levels or activity of RPL11 (Sun et al., 2010), and are thus indirect regulators of p53. Prior evidence suggests RPL37, RPS15, and RPS20 are detector RPs, as knockdown of RPL37 by siRNA (Llanos and Serrano, 2010) or mutation of RPS15 (Amsterdam et al., 2004) and RPS20 leads to p53 activation or tumorigenicity (McGowan et al., 2008). Nevertheless, our experiments suggest that they can also be seen as effector-type regulators of p53. We observed that RPL37, RPS15, and RPS20 can bind Mdm2, inhibit degradation of Mdm2 and p53, cause apoptosis and cell cycle arrest in G2, upregulate p21 and Puma mRNAs, and downregulate MdmX protein levels. Intriguingly, stable cell lines overexpressing GFP-RPL37 can arrest cells in G1 phase (Llanos and Serrano,

2010). The discrepancy between our results and theirs may due to the fact that the experiment we carried out used transient transfection while they were using cell lines harboring a GFP-tagged protein.

Currently, it is quite mysterious why so many RPs play seemingly redundant roles in regulating levels of p53. One conclusion to draw from the surfeit of RPs that can regulate the Mdm2-p53 axis is that ribosomal biogenesis is a hugely complex process and responding to interruptions in it is vitally important. It is possible that perturbation to the beginning, middle, or ending stages of ribosomal biogenesis generate specific stress signals that activate different RPs that go on to signal to the Mdm2-p53 axis. Also, different RPs may mediate p53 activation with different kinetics upon stress stimuli to ensure a proper cellular response. Finally, different RPs may target Mdm2 or p53 in different ways, such as inhibiting degradation of p53 protein or stimulating translation of p53 mRNA, as RPL26 does (Takagi et al., 2005).

As each RP is studied in more detail, it is possible that more differences will appear in the downstream consequences of their ability to activate p53. For example, we observed RPL37, RPS15, and RPS20 could stimulate G2 arrest, while RPL23, RPS7 and RPS25 have been shown to stimulate G1 arrest (Dai et al., 2004; Zhang et al., 2013; Zhu et al., 2009). Additionally, we have shown that RPL37, RPS15, and RPS20 differ from each other in their impacts on various p53 targets. A recent study showed knockdown of RPL37 in mouse embryonic stem cells or induced pluripotent stem cells led to the upregulation of multiple

p53 targets including p21, Mdm2, Pidd, Puma, Noxa, and Bax (Morgado-Palacin et al., 2012), while we found the ectopic expression of RPL37 in human osteosarcoma cells led to the selective upregulation of p21 and Puma but not Mdm2, Noxa or Bax. Furthermore, we found that only RPS15 and RPS20 have the ability to upregulate additional p53 targets, namely Mdm2 and miR-34a mRNAs. It remains to be seen if these RPs can be found at the promoters of these upregulated p53 targets, as was recently shown for RPL11 and various p53 targets following ActD treatment (Mahata et al., 2012). Since ChIP experiments rely on antibodies that can efficiently immunoprecipitate the protein of interest, and such antibodies are presently lacking for these RPs, those experiments will need to wait for the development of the appropriate reagents. RPS15 and RPS20 were also the only RPs that could co-immunoprecipitate with MdmX and downregulate MdmX protein levels, while RPL37 could downregulate both MdmX mRNA and protein levels without physically interacting with the MdmX protein. It is possible that RPS15 and RPS20 may function like RPL11 and regulate MdmX protein levels by enhancing Mdm2-mediated degradation (Gilkes et al., 2006), while RPL37 may use a mRNA-based mechanism to regulate MdmX levels. As these differences are explored in future studies, it is likely the model of segregating RPs into effectors versus detectors may need to be modified.

ACKNOWLEDGEMENTS

We are grateful to Ella Freulich for expert technical assistance and to members of the Prives laboratory for helpful discussions. We thank Hybrigenics (Paris) for supporting the Mdm2 yeast two hybrid screen analysis.

REFERENCES

Amsterdam A, Sadler KC, Lai K, Farrington S, Bronson RT, et al. (2004) Many ribosomal protein genes are cancer genes in zebrafish. PLoS Biol 2: E139.

Barkić M, Crnomarković S, Grabusić K, Bogetić I, Panić L, et al. (2009) The p53 tumor suppressor causes congenital malformations in Rpl24-deficient mice and promotes their survival. Mol Cell Biol 29: 2489-2504.

Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, et al. (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126: 107-120.

Biderman L, Poyurovsky MV, Assia Y, Manley JL, Prives C (2012) MdmX is required for p53 interaction with and full induction of the Mdm2 promoter after cellular stress. Mol Cell Biol 32: 1214-1225.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, et al. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282:1497-1501.

Bursac S, Brdovcak MC, Pfannkuchen M, Orsolic I, Golomb L, et al. (2012) Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. Proc Natl Acad Sci U S A 109: 20467-20472.

Chen D, Kastan MB (2010) 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes Dev 24: 2146-2156.

Chen D, Zhang Z, Li M, Wang W, Li Y, et al. (2007) Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene 26: 5029-5037.

Daftuar L, Zhu Y, Prives C (2010) Ribosomal protein L37 and the p53 network. Cell Cycle 9: 4259.

Dai MS, Lu H (2004) Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J Biol Chem 279: 44475-44482.

Dai MS, Zeng SX, Jin Y, Sun XX, David L, et al. (2004) Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Mol Cell Biol 24: 7654-7668.

Derezini M, Montanaro L, Trere D (2009) What the nucleolus says to a tumor pathologist. Histopathology 54: 753-762.

Fumagalli S, Di Cara A, Neb-Gulati A, Natt F, Schwemberger S, et al. (2009) Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rpL11-translation-dependent mechanism of p53 induction. Nat Cell Biol 11: 501-508.

Fumagalli S, Ivanenkov VV, Teng T, Thomas G (2012) Suprainduction of p53 by disruption of 40S and 60S ribosome biogenesis leads to the activation of a novel G2/M checkpoint. Genes Dev 26: 1028-1040.

Gazda HT, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, et al. (2008) Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. Am J Hum Genet 83: 769-780.

Gilkes DM, Chen L, Chen J (2006) MDMX regulation of p53 response to ribosomal stress. EMBO J 25: 5614-5625.

Gu J, Kawai H, Nie L, Kitao H, Wiederschain D, et al. (2002) Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. J Biol Chem 277: 19251-19254.

Hermeking H (2007) p53 enters the microRNA world. Cancer Cell 12: 414-418.

Jin A, Itahana K, O'Keefe K, Zhang Y (2004) Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Mol Cell Biol 24: 7669-7680.

Kawai H, Wiederschain D, Kitao H, Stuart J, Tsai KK, et al. (2003) DNA damage-induced MDMX degradation is mediated by MDM2. J Biol Chem 278: 45946-45953.

Kruse JP, Gu W (2009) Modes of p53 regulation. Cell 137: 609-622.

Levine AJ, Oren M (2009) The first 30 years of p53: growing ever more complex. Nat Rev Cancer 9: 749-758.

Lindström MS, Jin A, Deisenroth C, White Wolf G, Zhang Y (2007) Cancer-associated mutations in the MDM2 zinc finger domain disrupt ribosomal protein interaction and attenuate MDM2-induced p53 degradation. Mol Cell Biol 27: 1056-1068.

Lindström MS, Nistér M (2010) Silencing of ribosomal protein S9 elicits a multitude of cellular responses inhibiting the growth of cancer cells subsequent to p53 activation. PLoS One 5: e9578.

Linke K, Mace PD, Smith CA, Vaux DL, Silke J, et al. (2008) Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. Cell Death Differ 15: 841-848.

Llanos S, Serrano M (2010) Depletion of ribosomal protein L37 occurs in response to DNA damage and activates p53 through the L11/MDM2 pathway. Cell Cycle 9: 4005-4012.

Lohrum MA, Ludwig RL, Kubbutat MH, Hanlon M, Vousden KH (2003) Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell 3: 577-587.

Macias E, Jin A, Deisenroth C, Bhat K, Mao H, et al. (2010) An ARF-independent c-Myc-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 interaction. Cancer Cell 18: 231-243.

Mahata B, Sundqvist A, Xirodimas DP (2012) Recruitment of RPL11 at promoter sites of p53-regulated genes upon nucleolar stress through NEDD8 and in an Mdm2-dependent manner. Oncogene 31: 3060-3071.

Marechal V, Elenbaas B, Piette J, Nicolas JC, Levine AJ (1994) The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. Mol Cell Biol 14: 7414-7420.

McGowan KA, Li JZ, Park CY, Beaudry V, Tabor HK, et al. (2008) Ribosomal mutations cause p53-mediated dark skin and pleiotropic effects. Nat Genet 40: 963-970.

Momand J, Jung D, Wilczynski S, Niland J (1998) The MDM2 gene amplification database. Nucleic Acids Res 26: 3453-3459.

Morgado-Palacin L, Llanos S, Serrano M (2012) Ribosomal stress induces L11-and p53-dependent apoptosis in mouse pluripotent stem cells. Cell Cycle 11: 503-510.

Ofir-Rosenfeld Y, Boggs K, Michael D, Kastan MB, Oren M (2008) Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. Mol Cell 32: 180-189.

Ohtsuka T, Jensen MR, Kim HG, Kim KT, Lee SW (2004) The negative role of cyclin G in ATM-dependent p53 activation. Oncogene 23: 5405-5408.

Okamoto K, Li H, Jensen MR, Zhang T, Taya Y, et al. (2002) Cyclin G recruits PP2A to dephosphorylate Mdm2. Mol Cell 9: 761-771.

Peart MJ, Poyurovsky MV, Kass EM, Urist M, Verschuren EW, et al. (2010) APC/C(Cdc20) targets E2F1 for degradation in prometaphase. Cell Cycle 9: 3956-3964.

Shvarts A, Steegenga WT, Riteco N, van Laar T, Dekker P, et al. (1996) MDMX: a novel p53-binding protein with some functional properties of MDM2. EMBO J 15: 5349-5357.

Sun XX, Devine T, Challagundla KB, Dai MS (2011) Interplay between Ribosomal Protein S27a and MDM2 Protein in p53 Activation in Response to Ribosomal Stress. J Biol Chem 286: 22730-22741.

Sun XX, Wang YG, Xirodimas DP, Dai MS (2010) Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation. J Biol Chem 285: 25812-25821.

Takagi M, Absalon MJ, McLure KG, Kastan MB (2005) Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell 123: 49-63.

Tanimura S, Ohtsuka S, Mitsui K, Shirouzu K, Yoshimura A, et al. (1999) MDM2 interacts with MDMX through their RING finger domains. FEBS Lett 447: 5-9.

Toledo F, Wahl GM (2006) Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer 6: 909-923.

Tollini LA, Zhang Y (2012) p53 Regulation Goes Live-Mdm2 and MdmX Co-Star: Lessons Learned from Mouse Modeling. Genes Cancer 3: 219-225.

Vousden KH, Prives C (2009) Blinded by the Light: The Growing Complexity of p53. Cell 137: 413-431.

Wade M, Li YC, Wahl GM (2013) MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nat Rev Cancer 13: 83-96.

Xiong X, Zhao Y, He H, Sun Y (2011) Ribosomal protein S27-like and S27 interplay with p53-MDM2 axis as a target, a substrate and a regulator. Oncogene 30: 1798-1811.

Yadavilli S, Mayo LD, Higgins M, Lain S, Hegde V, et al. (2009) Ribosomal protein S3: A multi-functional protein that interacts with both p53 and MDM2 through its KH domain. DNA Repair (Amst) 8: 1215-1224.

Yamakuchi M, Ferlito M, Lowenstein CJ (2008) miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad Sci U S A 105: 13421-13426.

Zhang X, Wang W, Wang H, Wang MH, Xu W, et al. (2013) Identification of ribosomal protein S25 (RPS25)-MDM2-p53 regulatory feedback loop. Oncogene 32: 2782-2791.

Zhang Y, Wang J, Yuan Y, Zhang W, Guan W, et al. (2010) Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26. Nucleic Acids Res 38: 6544-6554.

Zhang Y, Wolf GW, Bhat K, Jin A, Allio T, et al. (2003) Ribosomal Protein L11 Negatively Regulates Oncoprotein MDM2 and Mediates a p53-Dependent Ribosomal-Stress Checkpoint Pathway. Mol Cell Biol 23: 8902-8912.

Zhou X, Hao Q, Liao J, Zhang Q, Lu H (2013) Ribosomal protein S14 unties the MDM2-p53 loop upon ribosomal stress. Oncogene 32: 388-396.

Zhu Y, Poyurovsky MV, Li Y, Biderman L, Stahl J, et al. (2009) Ribosomal Protein S7 Is Both a Regulator and a Substrate of MDM2. Mol Cell 35: 316-326.

FIGURE LEGENDS

Figure 2.1. RPL37, RPS15, and RPS20 interact with Mdm2. (a-c) Association of ectopically expressed RPs and Mdm2. H1299 cells were transfected with Flag-Mdm2 (1.2 μg), Myc-RP (1.2 μg), or both. (0.1 μg GFP was added as a control for transfection efficiency.) Cells were then lysed and subjected to immunoprecipitation and immunoblotting (IP) with the indicated antibodies as described. **(d) Association of endogenously expressed RPs and Mdm2.** SJSA cell lysates were subjected to immunoprecipitation with α-Mdm2, and co-immunoprecipitation of each RP was detected by immunoblotting with the relevant antibody. Since RPL37 and RPS20 run very close together, 2 independent blots are shown. In the left panel, RPL37 was blotted first and RPS15 second; in the right panel, RPS15 was blotted first and RPS20 was blotted second. Immunoblots in the right panel are taken from the same gel. **(e) Mapping sites of interaction between RPs and Mdm2.** H1299 cells were transfected with each Myc-RP (1.2 μg) and various amounts of each Flag-Mdm2 construct (full length; truncation 1-154; truncation 1-220; deletion 222-272; deletion 222-340; deletion 340-437; truncation 436-482) as shown in Figure 2.S2. Myc-RPs were immunoprecipitated with α-Myc, and co-immunoprecipitation of each RP and each Mdm2 construct was assayed by immunoblotting with α-Myc and α-Flag. Shown here is the summary of these binding assays (individual immunoblots are shown in Figure 2.S2). Mdm2 protein landmarks are depicted above, and regions bound by each indicated RPs is depicted below.

Figure 2.2. RPL37, RPS15, and RPS20 stabilize Mdm2. (a) Stabilization of ectopically expressed Mdm2 by RPs. H1299 cells were transfected with Flag-Mdm2 (1.2 μg) and Myc-RP (1.0-3.0 μg). (GFP was added as a control for transfection efficiency.) Mdm2 and RP levels were detected by immunoblotting with α-Myc and α-Flag. **(b-d) Inhibition of Mdm2 auto-ubiquitination by RPs.** H1299 cells were seeded in 60 mM tissue culture plates and transfected with HA-Ubiquitin (3.0 μg), Flag-Mdm2 (3.0 μg), and Myc-RP (8.0 μg for RPL37; 9.0 μg for RPS15 and RPS20). MG132 was added for 6 hours, and ubiquitinated Mdm2 species were assayed by immunoprecipitating with α-Flag and immunoblotting with α-HA. The asterisk indicates a non-specific band that runs below Flag-Mdm2. Inputs and IPs were run on separate gels.

Figure 2.3. RPL37, RPS15, and RPS20 increase levels of p53. (a) Stabilization of ectopically expressed p53 by RPs. U2OS cells were transfected with Flag-Mdm2 (1.2 μg), HA-p53 (0.3 μg), and Myc-RP (1.0-3.0 μg). (GFP was added as a control for transfection efficiency.) Ectopic Mdm2, p53, and RP levels were detected by immunoblotting with α-Flag, α-HA and α-Myc. **(b-d) Stabilization of endogenously expressed p53 by RPs.** U2OS cells were transfected with increasing amounts of Myc-RP (0-3.0 μg), and endogenous proteins were detected by immunoblotting with the indicated antibodies.

Figure 2.4. RPL37, RPS15, and RPS20 stabilize p53 protein. (a-c) Increase in half-life of p53 by RPs. U2OS cells were seeded in 35mM tissue culture plates and transfected with empty vector (3.0 μg) or Myc-RP (3.0 μg). Approximately 22 hours after the initial transfection, 100 μg/mL cycloheximide was added to the culture medium and cells were harvested at the indicated timepoints. **(d-f) Inhibition of Mdm2-mediated ubiquitination of p53 by RPs.** H1299 cells were seeded in 60mM tissue culture plates and transfected with HA-Ubiquitin (3.0 μg), p53 (0.75 μg), Flag-Mdm2 (7.5 μg), and Myc-RP (9.0 μg). MG132 was added for 6 hours, and ubiquitinated p53 species were assayed by immunoprecipitating with α-p53 and immunoblotting with α-HA. Inputs and IPs were run on separate gels.

Figure 2.5. Ectopic expression of RPL37, RPS15, and RPS20 increases cell death and G2 arrest. (a-d) Increase in Sub-G1 and G2 by RPs. U2OS cells were seeded in 60mM tissue culture plates and transfected with increasing amounts of Myc-RP (0-7.5 μg). Cell cycle analysis was carried out as described and normalized to the 0 μg Myc-RP control. The average of at least 3 independent experiments is plotted, and asterisks indicate where significant changes were observed in the cell cycle profile (* = $p<0.05$; ** = $p<0.01$; $n \geq 3$).

Figure 2.6. Ectopic expression of RPL37, RPS15, and RPS20 induces specific p53 target genes. (a-d) Increase in some p53 target genes by RPs.

U2OS cells were seeded in 60mM tissue culture plates and transfected with increasing amounts of Myc-RP (0-7.5 μg). Relative expression of each gene was determined in triplicate by quantitative RT-PCR and normalized to GAPDH. A representative experiment is plotted, and significant changes in mRNA levels were calculated using student's t-test (* = $p<0.05$; ** = $p<0.01$; $n \geq 3$).

Figure 2.7. RPL37, RPS15, and RPS20 bind to and regulate MdmX levels. (a-c) Association of RPs and MdmX. H1299 cells were transfected with HA-MdmX (0.5 μg), Myc-RP (1.2 μg), or both. (GFP was added as a control for transfection efficiency.) Ectopic MdmX and RP levels were detected by immunoblotting with α-MdmX and α-Myc. Inputs and IPs were run on separate gels. **(d-f) Decrease in MdmX protein by RPs.** U2OS cells were transfected with increasing amounts of Myc-RP (0-2.0 μg). Endogenous proteins were detected by immunoblotting with the indicated antibodies. **(g) Decrease in MdmX mRNA by RPL37.** U2OS cells were seeded in 60mM tissue culture plates and transfected with Myc-RP (0-5.0 μg). Relative expression of MdmX mRNA was determined by quantitative RT-PCR and normalized to GAPDH. A representative experiment is plotted, and significant changes in mRNA levels were calculated using student's t-test (* = $p<0.05$; ** = $p<0.01$; $n \geq 3$).

Figure 2.8. Knockdown of RPL37, RPS15, and RPS20 activate p53. (a-c) Increase in p53 and p21 by RPs. U2OS cells were transfected with siRNA

targeting RPL37, RPS15, or RPS20 as indicated (0 nM, 50 nM, 100 nM, 200 nM). Cells were harvested and lysates were subjected to immunoblotting for Mdm2, MdmX, p53, p21, and the indicated RPs with the relevant antibodies. Immunoblots in panel (c) are taken from the same gel.

SUPPORTING INFORMATION MATERIALS AND METHODS

Immunofluorescent microscopy

H1299 cells were transfected with Flag-Mdm2 and Myc-RPs as indicated. 24 hours after transfection, cells were fixed and immunostaining were carried out as previously described in [Karni-Schmidt O, Friedler A, Zupnick A, McKinney K, Mattia M, et al. (2007) Energy-dependent nucleolar localization of p53 in vitro requires two discrete regions within the p53 carboxyl terminus. Oncogene 26: 3878-3891]. Rabbit polyclonal anti-Myc (Sigma) and mouse monoclonal anti-Flag (Sigma) antibodies were used to detect Myc-RPs and Flag-Mdm2, respectively. Anti-rabbit Alexa Fluor 594 (Molecular Probes) and anti-mouse Alexa Fluor 488 (Molecular Probes) were used as secondary antibodies. Images were analyzed by confocal laser scanning microscopy (Model 1x81, Olympus) using Fluoview software (Olympus).

SUPPORTING INFORMATION LEGENDS

Figure 2.S1. RPL37, RPS15, and RPS20 interact with Mdm2 in U2OS cells. (a-c) U2OS cells were transfected with Flag-Mdm2 (1.2 μg), Myc-RP (1.2 μg), or both. (GFP was added as a control for transfection efficiency.) Cells were then lysed and subjected to immunoprecipitation and immunoblotting (IP) with the indicated antibodies as described.

Figure 2.S2. RPL37, RPS15, and RPS20 interact with the central region of Mdm2. (a) H1299 cells were transfected with Myc-RPL37 (1.2 μg), Flag-Mdm2 full length (1.2 μg), Flag-Mdm2 truncation 1-220 (0.1 μg), Flag-Mdm2 deletion 222-272 (1.2 μg), Flag-Mdm2 deletion 222-340 (1.1 μg), Flag-Mdm2 deletion 340-437 (0.1 μg), and Flag-Mdm2 truncation 436-482 (0.5 μg). **(b)** H1299 cells were transfected with Myc-RPS15 (1.2 μg), Flag-Mdm2 full length (1.2 μg), Flag-Mdm2 truncation 1-154 (1.2 μg), Flag-Mdm2 truncation 1-220 (0.1 μg), Flag-Mdm2 deletion 222-272 (1.2 μg), Flag-Mdm2 deletion 222-340 (0.5 μg), Flag-Mdm2 deletion 340-437 (0.25 μg), and Flag-Mdm2 truncation 436-482 (0.25 μg). **(c)** H1299 cells were transfected with Myc-RPS20 (1.2 μg), Flag-Mdm2 full length (1.2 μg), Flag-Mdm2 truncation 1-154 (0.04 μg), Flag-Mdm2 truncation 1-220 (0.02 μg), Flag-Mdm2 deletion 222-272 (1.95 μg), Flag-Mdm2 deletion 222-340 (1.0 μg), Flag-Mdm2 deletion 340-437 (0.1 μg), and Flag-Mdm2 truncation 436-482 (0.3 μg). **(d)** H1299 cells were transfected with Myc-RPS20 (1.2 μg) and equal amounts of each Flag-Mdm2 construct (1.2 μg of full length, truncation 1-

220, deletion 222-340, deletion 340-437, truncation 438-483). **(e)** H1299 cells were transfected with Myc-RPS20 (1.2 μg), Flag-Mdm2 full length (1.2 μg), Flag-Mdm2 truncation 1-220 (0.3 μg), Flag-Mdm2 deletion 222-340 (0.9 μg), Flag-Mdm2 deletion 340-437 (0.3 μg), and Flag-Mdm2 truncation 436-482 (0.4 μg). For all transfections, Myc-RPs were immunoprecipitated with α-Myc and co-immunoprecipitation of each RP and each Mdm2 construct was assayed by immunoblotting with α-Myc and α-Flag. In panels (b)-(d), inputs and IPs were run on separate gels.

Figure 2.S3. RPL37, RPS15, and RPS20 stabilization of Mdm2 seen by immunofluorescent microscopy. H1299 cells were grown on coverslips in 35mM tissue culture plates and transfected with Flag-Mdm2 (1.2 μg), Myc-RP (1.2 μg), or both. Immunofluorescent staining was carried out as described.

Figure 2.S4. RPL37, RPS15, and RPS20 do not upregulate mRNA levels of Ccng1, Bax, Noxa, or Tigar. U2OS cells were seeded in 60mM tissue culture plates and transfected with increasing amounts of Myc-RP (0-7.5 μg). Relative expression of each gene was determined in triplicate by quantitative RT-PCR and normalized to GAPDH. A representative experiment is plotted, and significant changes in mRNA levels were calculated using student's t-test (* = $p<0.05$; ** = $p<0.01$; $n > 3$).

Table 2.T1. siRNA sequences. The sequences for the siRNAs used are provided.

Table 2.T2. qRT-PCR sequences. The primer sequences for the qRT-PCR reactions performed are provided.

FIGURE 2.1

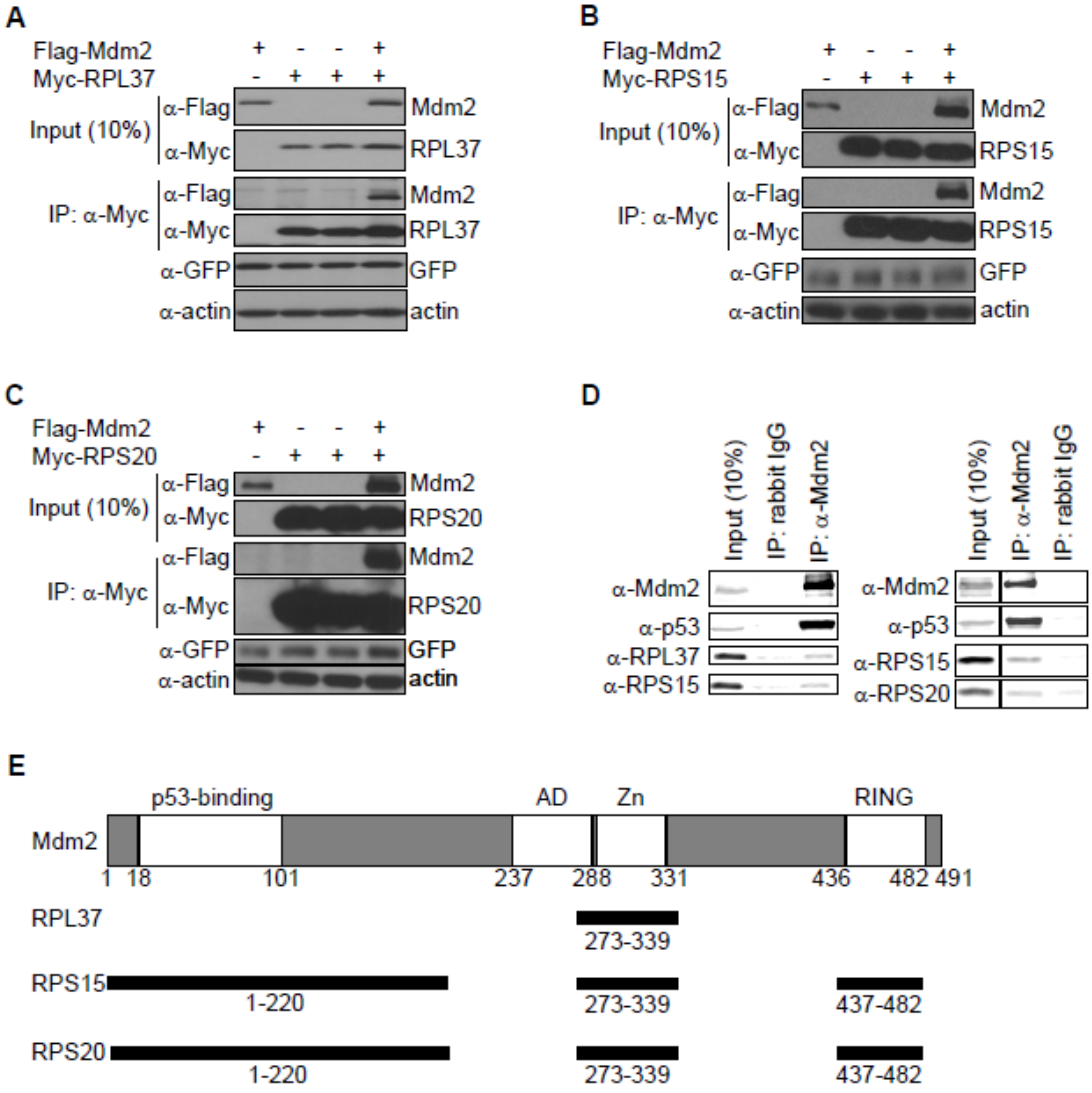

A
Flag-Mdm2
Myc-RPL37
Input (10%)
α-Flag
α-Myc
Mdm2
RPL37
IP: α-Myc
α-GFP
GFP
α-actin
actin
B
Flag-Mdm2
Myc-RPS15
RPS15
C
Myc-RPS20
RPS20
D
Input (10%)
IP: rabbit IgG
IP: α-Mdm2
α-Mdm2
α-p53
α-RPL37
α-RPS15
α-RPS20
E
p53-binding
AD
Zn
RING
Mdm2
1 18 101 237 288 331 436 482 491
RPL37
273-339
RPS15
1-220
273-339
437-482
RPS20
1-220
273-339
437-482

FIGURE 2.2

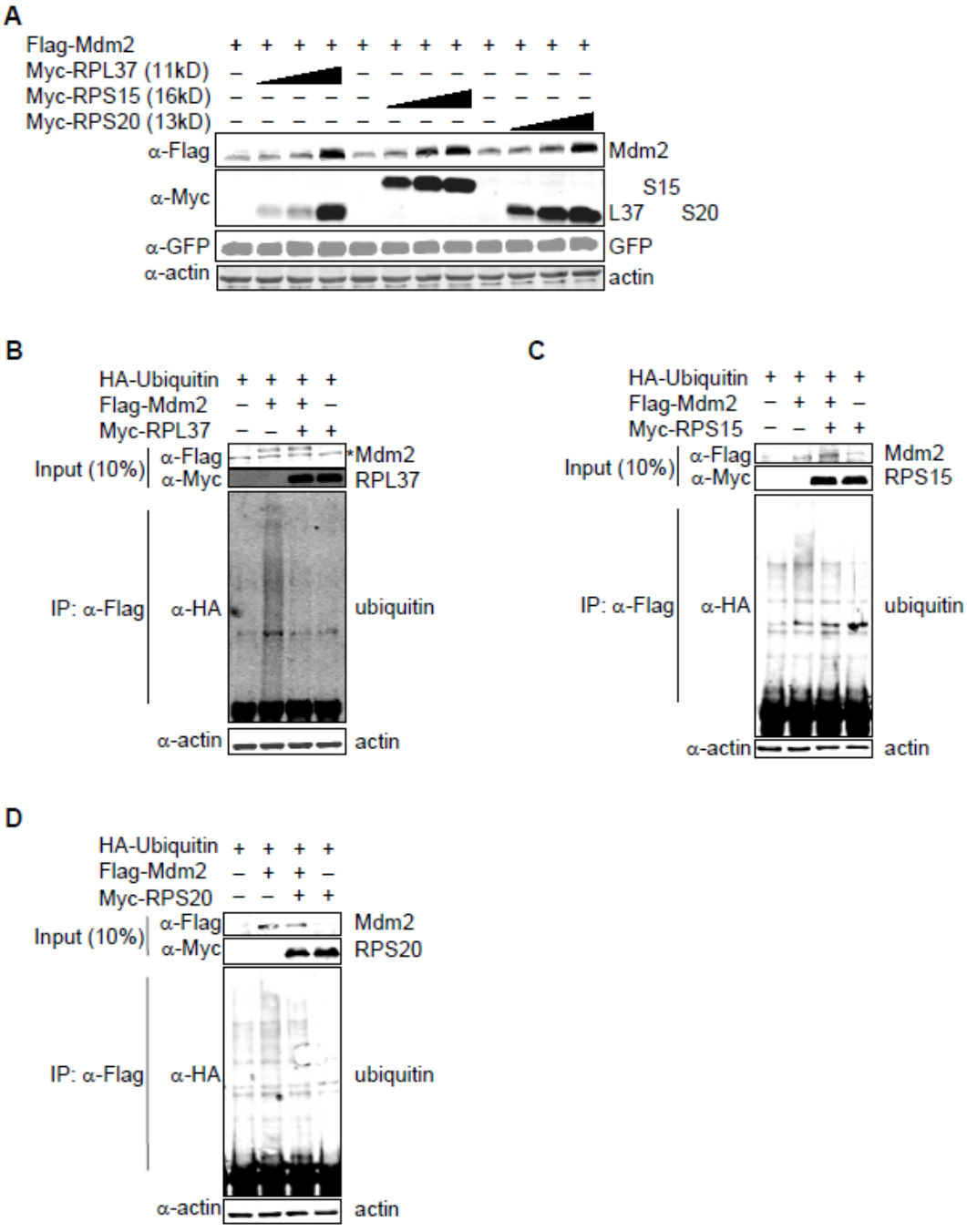

A
Flag-Mdm2
Myc-RPL37 (11kD)
Myc-RPS15 (16kD)
Myc-RPS20 (13kD)
α-Flag
α-Myc
α-GFP
α-actin
Mdm2
S15
L37
S20
GFP
actin
B
HA-Ubiquitin
Flag-Mdm2
Myc-RPL37
Input (10%)
α-Flag
α-Myc
*Mdm2
RPL37
IP: α-Flag
α-HA
ubiquitin
α-actin
actin
C
HA-Ubiquitin
Flag-Mdm2
Myc-RPS15
Input (10%)
α-Flag
α-Myc
Mdm2
RPS15
IP: α-Flag
α-HA
ubiquitin
α-actin
actin
D
HA-Ubiquitin
Flag-Mdm2
Myc-RPS20
Input (10%)
α-Flag
α-Myc
Mdm2
RPS20
IP: α-Flag
α-HA
ubiquitin
α-actin
actin

FIGURE 2.3

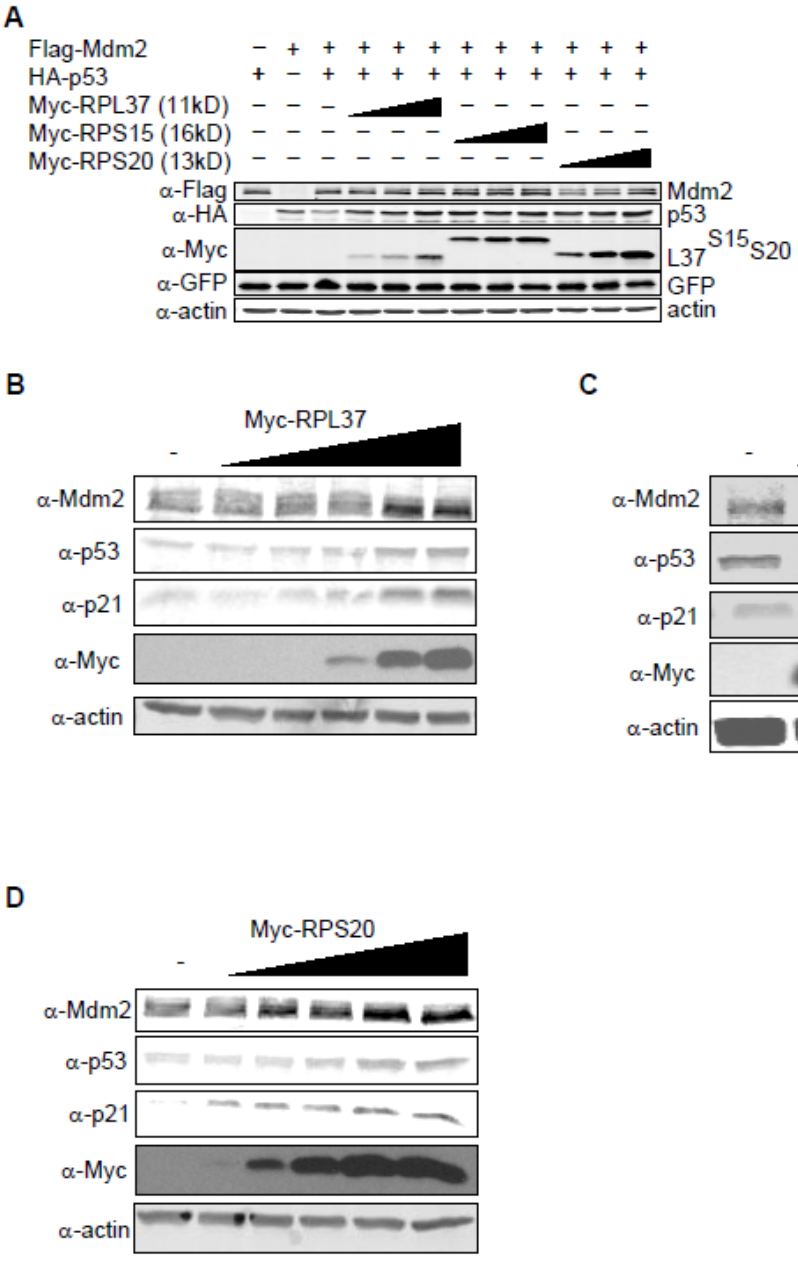

A
Flag-Mdm2
HA-p53
Myc-RPL37 (11kD)
Myc-RPS15 (16kD)
Myc-RPS20 (13kD)
α-Flag
α-HA
α-Myc
α-GFP
α-actin
Mdm2
p53
L37 S15 S20
GFP
actin
B
Myc-RPL37
α-Mdm2
α-p53
α-p21
α-Myc
α-actin
C
Myc-RPS15
α-Mdm2
α-p53
α-p21
α-Myc
α-actin
D
Myc-RPS20
α-Mdm2
α-p53
α-p21
α-Myc
α-actin

FIGURE 2.4

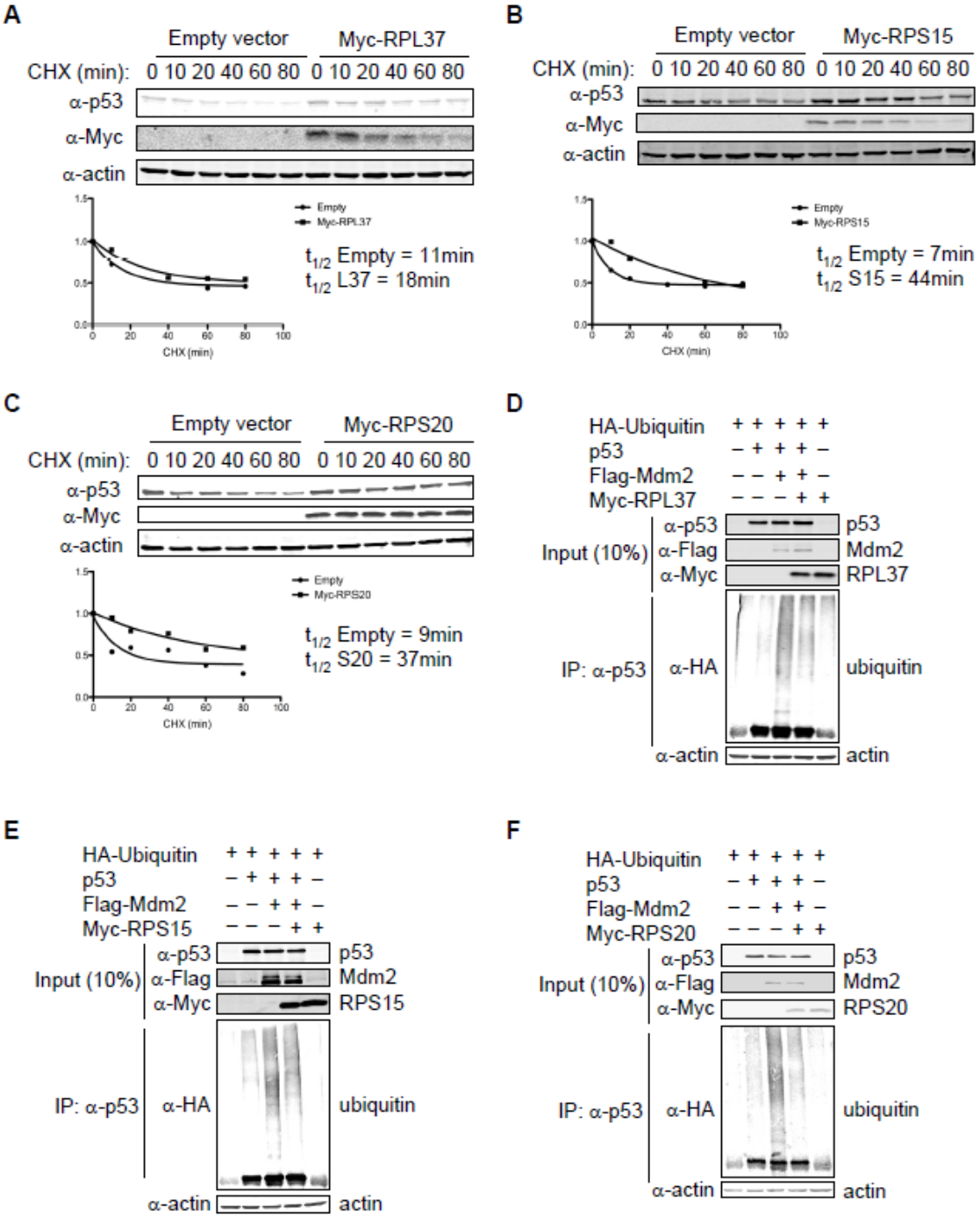

A
Empty vector
Myc-RPL37
CHX (min): 0 10 20 40 60 80 0 10 20 40 60 80
α-p53
α-Myc
α-actin
Empty
Myc-RPL37
CHX (min)
t1/2 Empty = 11min
t1/2 L37 = 18min
B
Empty vector
Myc-RPS15
CHX (min): 0 10 20 40 60 80 0 10 20 40 60 80
α-p53
α-Myc
α-actin
Empty
Myc-RPS15
CHX (min)
t1/2 Empty = 7min
t1/2 S15 = 44min
C
Empty vector
Myc-RPS20
CHX (min): 0 10 20 40 60 80 0 10 20 40 60 80
α-p53
α-Myc
α-actin
Empty
Myc-RPS20
CHX (min)
t1/2 Empty = 9min
t1/2 S20 = 37min
D
HA-Ubiquitin + + + + +
p53 – + + + –
Flag-Mdm2 – – + + –
Myc-RPL37 – – – + +
Input (10%)
α-p53
α-Flag
α-Myc
p53
Mdm2
RPL37
IP: α-p53
α-HA
ubiquitin
α-actin
actin
E
HA-Ubiquitin + + + + +
p53 – + + + –
Flag-Mdm2 – – + + –
Myc-RPS15 – – – + +
Input (10%)
α-p53
α-Flag
α-Myc
p53
Mdm2
RPS15
IP: α-p53
α-HA
ubiquitin
α-actin
actin
F
HA-Ubiquitin + + + + +
p53 – + + + –
Flag-Mdm2 – – + + –
Myc-RPS20 – – – + +
Input (10%)
α-p53
α-Flag
α-Myc
p53
Mdm2
RPS20
IP: α-p53
α-HA
ubiquitin
α-actin
actin

FIGURE 2.5

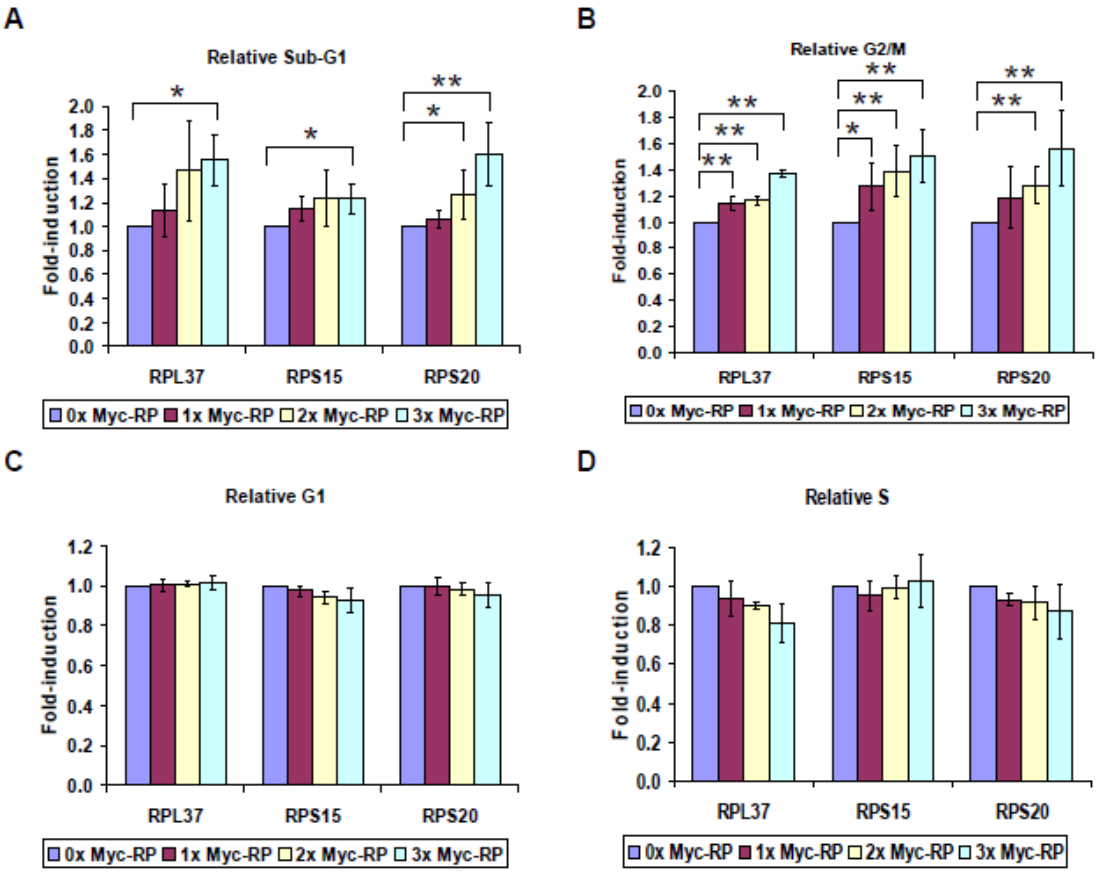

A
Relative Sub-G1
Fold-induction
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP
3x Myc-RP
B
Relative G2/M
C
Relative G1
D
Relative S

FIGURE 2.6

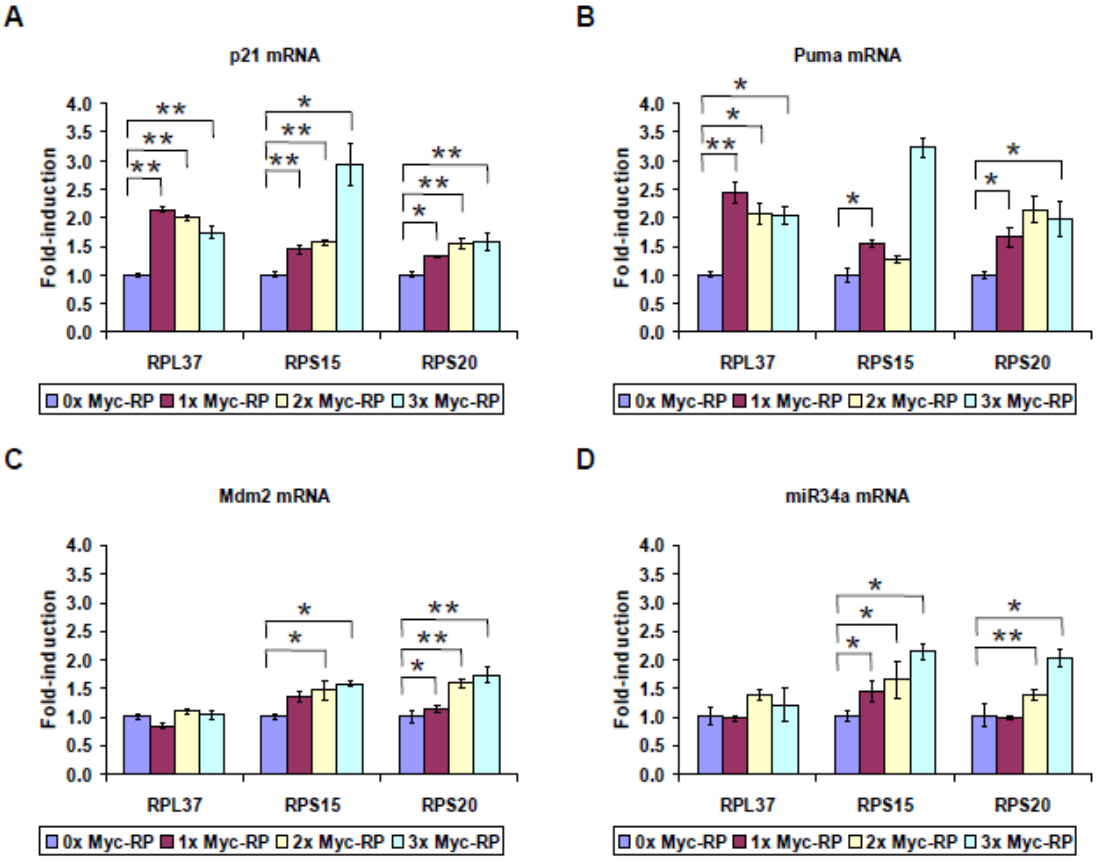

A
p21 mRNA
B
Puma mRNA
C
Mdm2 mRNA
D
miR34a mRNA
Fold-induction
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP
3x Myc-RP

FIGURE 2.7

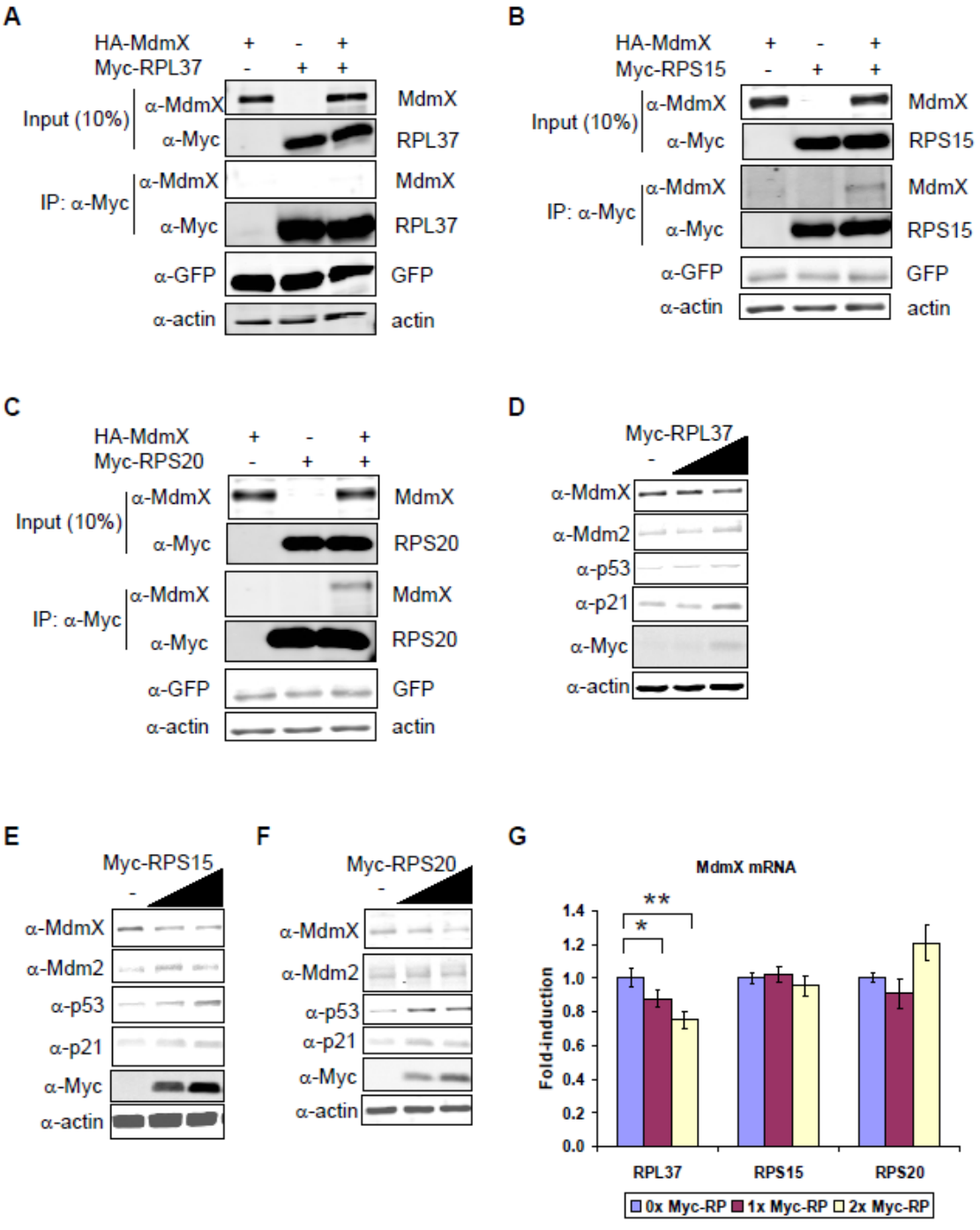

A
HA-MdmX + - +
Myc-RPL37 - + +
Input (10%)
α-MdmX
MdmX
α-Myc
RPL37
IP: α-Myc
α-MdmX
MdmX
α-Myc
RPL37
α-GFP
GFP
α-actin
actin
B
HA-MdmX + - +
Myc-RPS15 - + +
Input (10%)
α-MdmX
MdmX
α-Myc
RPS15
IP: α-Myc
α-MdmX
MdmX
α-Myc
RPS15
α-GFP
GFP
α-actin
actin
C
HA-MdmX + - +
Myc-RPS20 - + +
Input (10%)
α-MdmX
MdmX
α-Myc
RPS20
IP: α-Myc
α-MdmX
MdmX
α-Myc
RPS20
α-GFP
GFP
α-actin
actin
D
Myc-RPL37
-
α-MdmX
α-Mdm2
α-p53
α-p21
α-Myc
α-actin
E
Myc-RPS15
-
α-MdmX
α-Mdm2
α-p53
α-p21
α-Myc
α-actin
F
Myc-RPS20
-
α-MdmX
α-Mdm2
α-p53
α-p21
α-Myc
α-actin
G
MdmX mRNA
**
*
Fold-induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP

FIGURE 2.8

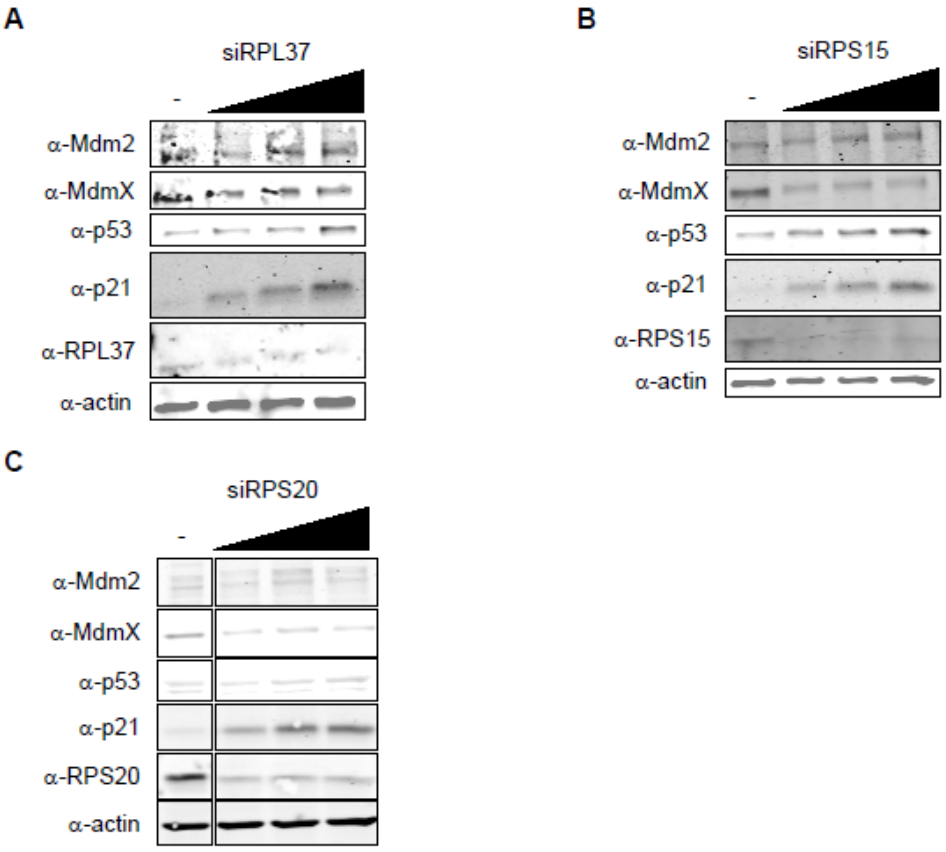

A
siRPL37
-
α-Mdm2
α-MdmX
α-p53
α-p21
α-RPL37
α-actin
B
siRPS15
-
α-Mdm2
α-MdmX
α-p53
α-p21
α-RPS15
α-actin
C
siRPS20
-
α-Mdm2
α-MdmX
α-p53
α-p21
α-RPS20
α-actin

FIGURE 2.S1

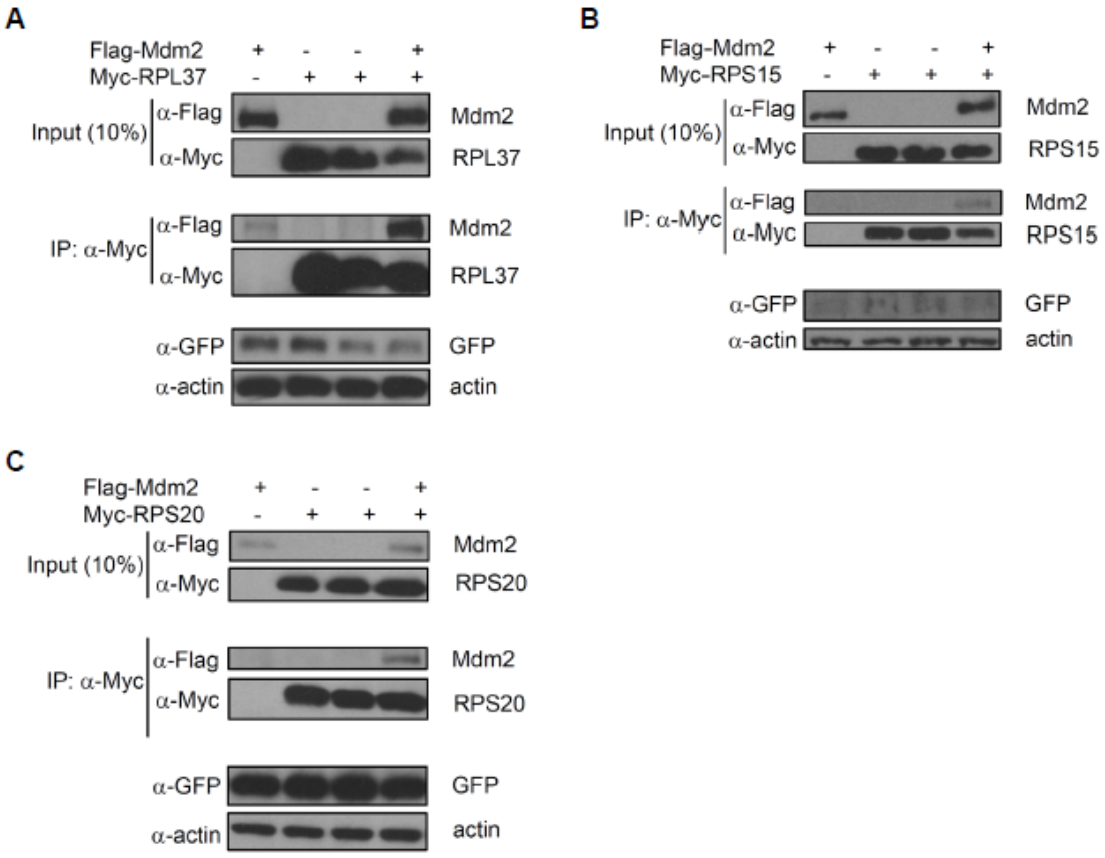

A
Flag-Mdm2 + - - +
Myc-RPL37 - + + +
Input (10%)
α-Flag
α-Myc
Mdm2
RPL37
IP: α-Myc
α-Flag
α-Myc
Mdm2
RPL37
α-GFP
α-actin
GFP
actin
B
Flag-Mdm2 + - - +
Myc-RPS15 - + + +
Input (10%)
α-Flag
α-Myc
Mdm2
RPS15
IP: α-Myc
α-Flag
α-Myc
Mdm2
RPS15
α-GFP
α-actin
GFP
actin
C
Flag-Mdm2 + - - +
Myc-RPS20 - + + +
Input (10%)
α-Flag
α-Myc
Mdm2
RPS20
IP: α-Myc
α-Flag
α-Myc
Mdm2
RPS20
α-GFP
α-actin
GFP
actin

FIGURE 2.S2

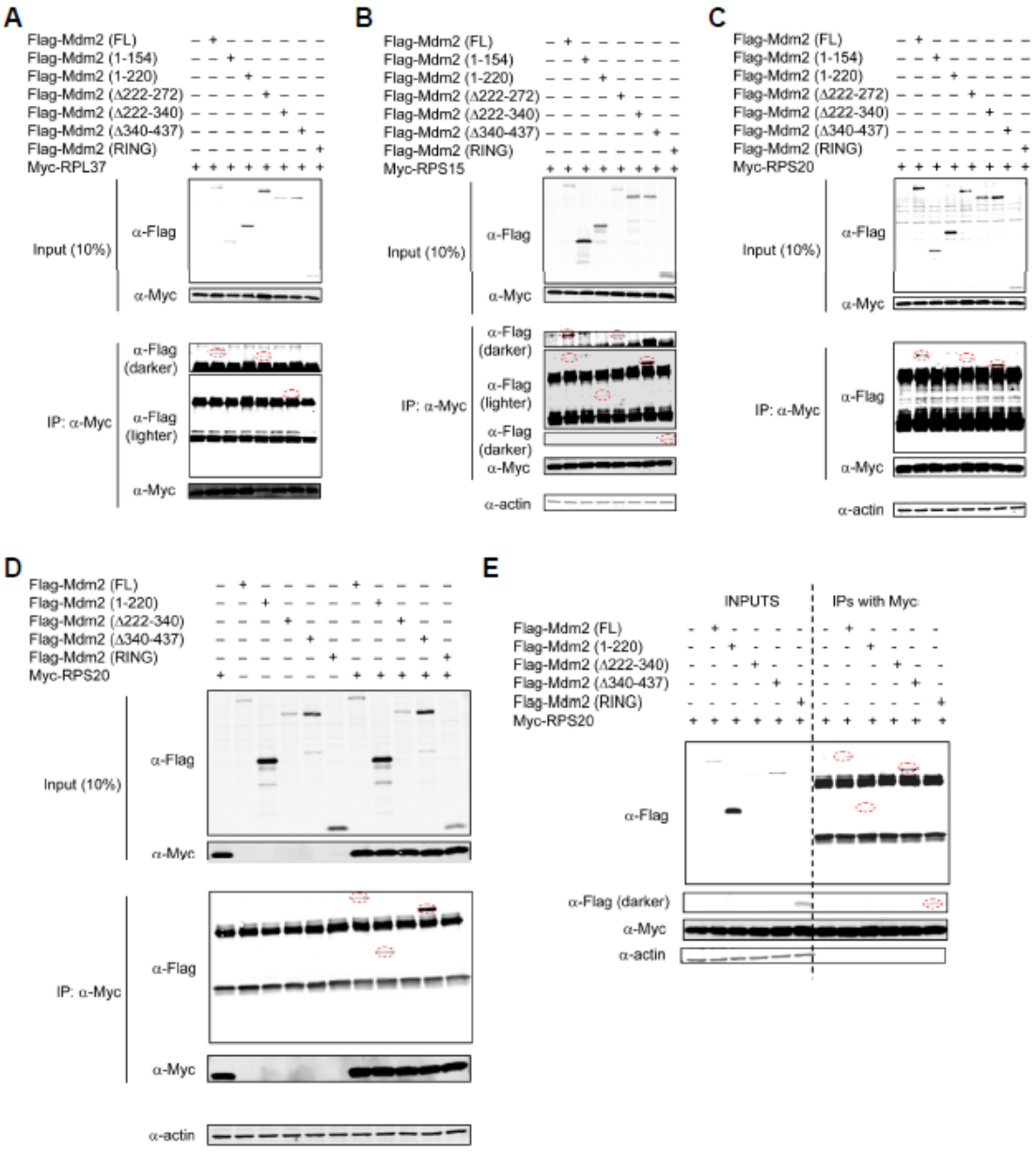

A
Flag-Mdm2 (FL)
Flag-Mdm2 (1-154)
Flag-Mdm2 (1-220)
Flag-Mdm2 (Δ222-272)
Flag-Mdm2 (Δ222-340)
Flag-Mdm2 (Δ340-437)
Flag-Mdm2 (RING)
Myc-RPL37
Input (10%)
α-Flag
α-Myc
IP: α-Myc
α-Flag (darker)
α-Flag (lighter)
α-Myc
B
Flag-Mdm2 (FL)
Flag-Mdm2 (1-154)
Flag-Mdm2 (1-220)
Flag-Mdm2 (Δ222-272)
Flag-Mdm2 (Δ222-340)
Flag-Mdm2 (Δ340-437)
Flag-Mdm2 (RING)
Myc-RPS15
Input (10%)
α-Flag
α-Myc
IP: α-Myc
α-Flag (darker)
α-Flag (lighter)
α-Flag (darker)
α-Myc
α-actin
C
Flag-Mdm2 (FL)
Flag-Mdm2 (1-154)
Flag-Mdm2 (1-220)
Flag-Mdm2 (Δ222-272)
Flag-Mdm2 (Δ222-340)
Flag-Mdm2 (Δ340-437)
Flag-Mdm2 (RING)
Myc-RPS20
Input (10%)
α-Flag
α-Myc
IP: α-Myc
α-Flag
α-Myc
α-actin
D
Flag-Mdm2 (FL)
Flag-Mdm2 (1-220)
Flag-Mdm2 (Δ222-340)
Flag-Mdm2 (Δ340-437)
Flag-Mdm2 (RING)
Myc-RPS20
Input (10%)
α-Flag
α-Myc
IP: α-Myc
α-Flag
α-Myc
α-actin
E
INPUTS
IPs with Myc
Flag-Mdm2 (FL)
Flag-Mdm2 (1-220)
Flag-Mdm2 (Δ222-340)
Flag-Mdm2 (Δ340-437)
Flag-Mdm2 (RING)
Myc-RPS20
α-Flag
α-Flag (darker)
α-Myc
α-actin

FIGURE 2.S3

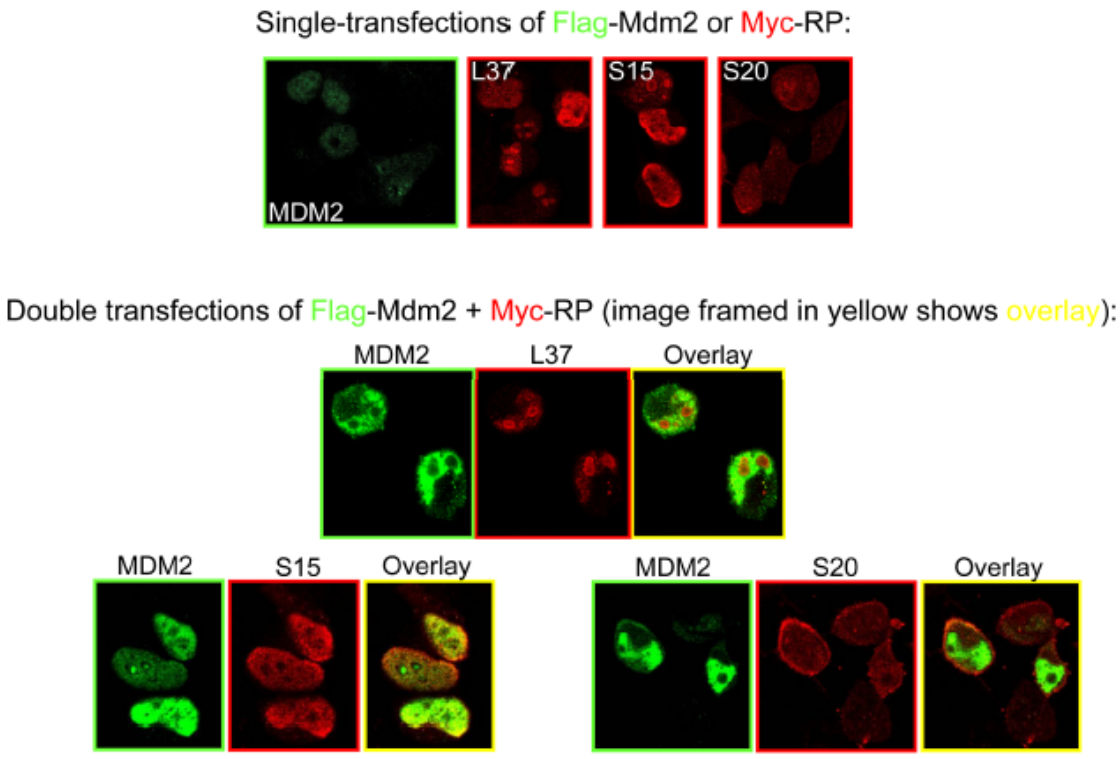

Single-transfections of Flag-Mdm2 or Myc-RP:
MDM2
L37
S15
S20
Double transfections of Flag-Mdm2 + Myc-RP (image framed in yellow shows overlay):
MDM2
L37
Overlay
MDM2
S15
Overlay
MDM2
S20
Overlay

FIGURE 2.S4

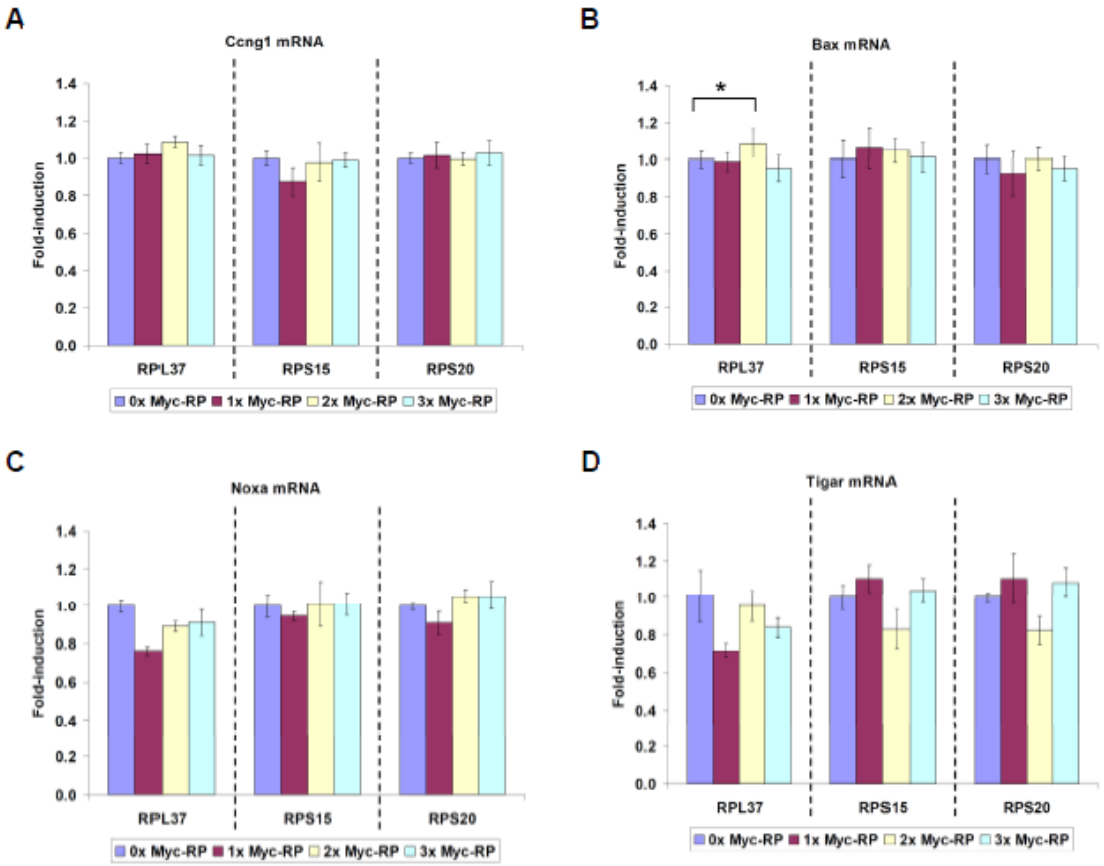

A
Ccng1 mRNA
Fold-induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP
3x Myc-RP
B
Bax mRNA
*
Fold-induction
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP
3x Myc-RP
C
Noxa mRNA
Fold-induction
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP
3x Myc-RP
D
Tigar mRNA
Fold-induction
RPL37
RPS15
RPS20
0x Myc-RP
1x Myc-RP
2x Myc-RP
3x Myc-RP

FIGURE 2.T1

siLuc target sequence: AACTTACGCTGAGTACTTCGA
siRPL37 target sequence: AAGTGAGTAGCTAACTTCCAA
siRPS15 target sequence: CAAGTAATGGCTCAGCTAATA
siRPS20 target sequence: CTGACTTGATAAGAGGCGCAA

FIGURE 2.T2

Bax	Forward: TGGAGCTGCAGAGGATGATTG
	Reverse: AAACATGTCAGCTGCCACTCG
Ccng1	Forward: TACCGCTGAGGAGCTGCAGT
	Reverse: CAGTTGTTGTCAGTACCTCTATCACTTG
GAPDH	Forward: TGCACCACCAACTGCTTAGC
	Reverse: GGCATGGACTGTGGTCATGAG
Mdm2	Forward: TTGGCGTGCCAAGCTTCTCT
	Reverse: TACCTGAGTCCGATGATTCC
MdmX	Forward: GCAAGAAATTTAACTCTCCAAGCAA
	Reverse: CTTTGAACAATCTGAATACCAATCCTT
miR34a	Forward: CTCGGTGACCACGCAGATC
	Reverse: GCAGGACTCCCGCAAAATC
Noxa	Forward: GCAAGAACGCTCAACCGAG
	Reverse: CAAATCTCCTGAGTTGAGTAGCACAC
p21	Forward: GGCGGCAGACCAGCATGACA
	Reverse: GCAGGGGGCGGCCAGGGTAT
Puma	Forward: CCTGGAGGGTCCTGTACAATCT
	Reverse: GCACCTAATTGGGCTCCATCT
TIGAR	Forward: CCAGGCTCGCAGCTTCA
	Reverse: GGTTTCGACTCCAGGTGCAA

CHAPTER 3

RPL36A is a negative regulator of the Mdm2-p53 axis

Lilyn Daftuar[1], Yan Zhu[1], Xavier Jacq[2] and Carol Prives[1,*]

[1] Department of Biological Sciences, Columbia University, New York, New York, United States of America

[2] Present address: MISSION Therapeutics Ltd, Cambridge, United Kingdom

* Corresponding Author: clp3@columbia.edu

ABSTRACT

p53 is an important tumor suppressor in cells that causes cell cycle arrest or apoptosis in response to many forms of stress. Its activity is kept in check by Mdm2, an E3 ubiquitin ligase that targets p53 for degradation. Research over the past decade has uncovered a ribosomal protein (RP)-Mdm2-p53 pathway that is critical for responding to ribosomal stress. All of the RPs that have been identified to date that participate in the pathway bind to Mdm2, inhibit its E3 ligase function, activate p53, and are necessary for a full p53 response to ribosomal stress. Here, we present evidence that a novel extra-ribosomal function exists for RPL36A. RPL36A interacts with Mdm2, but leads to lower levels of p53 when overexpressed rather than higher. Overexpression of RPL36A also attenuates the response to ribosomal stress, while knockdown accentuates it. Endogenous RPL36A also appears to move from a soluble state to an insoluble state in response to ribosomal stress, suggesting that unlike other ribosomal proteins, its removal from the Mdm2-p53 axis is essential for a full p53 response.

INTRODUCTION

p53 is an important tumor suppressor whose loss or mutation has been implicated in over half over the cases of human cancer (Levine and Oren, 2009). It is a stress-responsive sequence-specific transcription factor with a wide variety of targets that can mediate many cellular outcomes (Gomez-Lazaro et al., 2004). In the absence of stress, p53 levels and activity are kept in check by its negative regulator Mdm2. Mdm2 is a RING-type E3 ubiquitin ligase that ubiquitinates and targets p53 for nuclear export and proteasome-mediated degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Mdm2 can also directly bind to and inhibit p53's transactivation domain and prevent target gene activation. In response to stress, p53 can upregulate targets such as p21 and Puma to cause cell cycle arrest or apoptosis. p53 has many additional targets, one of which is Mdm2. The transcription of Mdm2 by p53 creates a negative feedback loop that shuts off p53 activity once the stress has passed.

Many forms of stress have been shown to break the Mdm2-p53 interaction and upregulate p53 levels and activity. One such stress is ribosomal stress, also known as nucleolar stress. Ribosomal biogenesis is a complex process that takes place in the nucleolus, and disruptions to any of its steps can trigger stress that signals to p53 (Boisevert et al., 2007). Over the past decade, multiple groups have shown the existence of a ribosomal protein (RP)-Mdm2-p53 pathway that is important for the response to ribosomal stress (Deisenroth and Zhang, 2010). There are approximately 79 eukaryotic ribosomal proteins (33 RPS's in the 40S

small subunit and 46 RPL's in the 60S large subunit), and 12 of them have been shown to interact with Mdm2, inhibit its E3 ubiquitin ligase activity, stabilize p53 levels and increase p53 activity. In order of discovery, they are RPL11 (Lohrum et al., 2003; Zhang et al., 2003), RPL23 (Dai et al., 2004; Jin et al., 2004), RPL5 (Dai and Lu, 2004), RPS7 (Chen et al., 2007; Zhu et al., 2009), RPL26 (Zhang et al., 2010), RPS27 and RPS27L (Xiong et al., 2011), RPS27A (Sun et al., 2011), RPS14 (Zhou et al., 2013), RPS25 (Zhang et al., 2013), RPS26 (Cui et al., 2013), RPL37, RPS15, and RPS20 (Daftuar et al., 2013). One of those 12, RPL26, has an additional mechanism for stimulating p53 activity; it can also increase p53 levels by augmenting translation of its mRNA (Takagi et al., 2005; Chen and Kastan, 2010).

Thus, the accumulated evidence suggests RPs have extra-ribosomal functions as tumor suppressors in the Mdm2-p53 axis. However, oncogenic extra-ribosomal roles for a few RPs have been identified. Overexpression of RPS3A in 3T3 cells led to transformation and tumor development in nude mice (Naora et al., 1998), although the mechanism remains unclear. And although RPL23 can increase p53 as described above, it can also indirectly promote Myc activation and cellular proliferation (Wanzel et al., 2008), suggesting its role *in vivo* may be more complex than other RPs that have only been shown to activate p53. However, no RPs have previously been shown to function as oncogenes through the Mdm2-p53 axis.

A yeast two-hybrid screen using Mdm2 as the bait identified RPL36A and its closely related homolog RPL36AL as potential Mdm2 interactors. RPL36A is found on the X chromosome in humans, and like the other sex-linked ribosomal proteins (RPL39 and RPL10), an autosomal version of RPL36A exists called RPL36AL. The autosomal versions were likely retrotransposed from the X-linked genes and may have evolved to compensate for the reduced dosage of X-linked RP genes (Uechi et al., 2002). While the sequences of RPL36A and RPL36AL differ somewhat at the DNA level, they only differ at the protein level by 1 amino acid at position 38 (K in RPL36A; R in RPL36AL), and they can substitute for each other in the ribosome (Baouz et al., 2009). RPL36A is a highly basic 60S large subunit RP that is conserved among eukaryotic 80S ribosomes but absent in prokaryotic 70S ribosomes. Early studies suggested RPL36A is localized to the peptidyl-tRNA binding site of translating ribosomes, alongside 5 other RPs (Fabijanski and Pellegrini, 1981). Later studies concurred that RPL36A is found deep within the ribosome, buried beneath other RPs and obscured from the surface by 28S rRNA (Marion and Marion, 1987).

A few subtle hints existed previously for extra-ribosomal functions for RPL36A. One study found its mRNA, along with 22 other RPs, was downregulated during neuronal differentiation (Bevort and Leffers, 2000). Another study showed decreased levels of RPL36A protein in 2-week old male rats whose mothers had experienced chronic exposure to nitric oxide (Wesseling et al., 2011). More recently, it was shown that HDAC6 relocalizes in

keratinocytes treated with arsenite and interacts with both RPL36A and RPS6 outside the nucleus (Kappeler et al., 2012). Related to cancer, one study found RPL36A levels are upregulated in hepatocellular carcinoma (Kim et al., 2004), and another found that it relocalizes to the surface of cells during doxorubicin-induced apoptosis (Nishida et al., 2002). RPL36A+/- zebrafish have impaired growth and a predisposition for forming nerve sheath tumors (Amsterdam et al., 2004). We present evidence here that RPL36A has a novel extra-ribosomal role as an oncogene that can inhibit p53.

MATERIALS AND METHODS

Plasmids and siRNA

Flag-Mdm2 and its deletion constructs, GFP, HA-p53, HA-ubiquitin, p53, pcDNA3-Myc were described previously (Zhu et al., 2009). RPL36A was cloned into the pcDNA3-Myc mammalian expression vector using the following forward and reverse primers: 5'-GCGGATCCAGTTAACGTCCCTAAAAC-3' and 5'-GCGAATTCTTAGAACTGGATCACTTG-3'. RPL36AL was sub-cloned from RPL36A using the following forward and reverse primers: 5'-CTGTACGCCCAGGGAAGGCGGCGTTATGACAGG-3' and 5'-CCTGTCATAACGCCGCCTTCCCTGGGCGTACAG-3' (QuikChange Site-Directed Mutagenesis Kit, Agilent). Note the sub-cloned RPL36AL has the amino acid sequence of RPL36AL but not its exact DNA sequence. siRPL36A and siRPL36AL sequences were pre-designed by the manufacturer (Hs_RPL36A_9 SI04164454, Hs_RPL36A_10 SI04302088, Hs_RPL36AL_1 SI00706419, Hs_RPL36AL_2 SI00706426, Hs_RPL36AL_7 SI04250078, Qiagen), while siLuc (siRNA against luciferase) was previously described (Peart et al., 2010).

Cell culture, transfection, and drugs

H1299 lung carcinoma, SJSA osteosarcoma, and U2OS osteosarcoma cell lines were cultured as described previously (Zhu et al., 2009). Transfections were carried out as described previously (Daftuar et al., 2013). Briefly, DNA transfections were carried out for 24 hours with Lipofectamine 2000 according to

the manufacturer's instructions (Invitrogen), and siRNA transfections were carried out for 72 hours with DharmaFECT 1 according to the manufacturer's instructions (Thermo Scientific). The following drugs were added prior to harvesting for the indicated amounts of time: actinomycin D (Sigma), cycloheximide (Sigma), 5-fluorouracil (Sigma), or MG132 (Calbiochem).

Gel electrophoresis, Coomassie Blue staining, and immunoblotting

Gel electrophoresis and immunoblotting were carried out as described previously (Daftuar et al., 2013). Briefly, cell pellets were lysed with Lysis Buffer (25 mM Tris-Hcl pH 7.5, 137 mM NaCl, 2.7 mM KCl, and 0.5% Igepal CA-630 supplemented with 50 nM PMSF and inhibitor cocktail containing 100uM Benzamidine, 300ug/uL Leupeptin, 100mg/mL Bacitracin, and 1mg/mL a2-macroglobulin) and unless indicated otherwise, cell lysates were cleared by spinning at 4,000 rpm for 10 minutes. Equivalent amounts of protein were loaded onto polyacrylamide gels, and gels were either stained with Coomassie Brilliant Blue G-250 solution (Fisher) or transferred onto nitrocellulose membranes (Bio-Rad). For immunoblots, membranes were probed using the following antibodies: anti-actin (A2066, Sigma); anti-Flag (M2, Sigma); anti-GFP (B2, Santa Cruz Biotechnology); anti-HA (16B12, Covance); anti-Mdm2 (a mixture of 3G5, 4B11, and 5B10 hybridomas); anti-Myc (9E10, Santa Cruz Biotechnology or C3956, Sigma); anti-p21 (C19, Santa Cruz Biotechnology); anti-p53 (a mixture of 1801 and D01 hybridomas); anti-RPL36A (43A, Santa Cruz Biotechnology).

Membranes were visualized either using chemiluminescence (ECL, GE Healthcare) or fluorescence (IRDye, LI-COR Biosciences).

Immunoprecipitations

H1299 or U2OS cells were transfected with Myc-RPL36A, Flag-Mdm2, and various Flag-Mdm2 truncation and deletion constructs as indicated. Anti-Myc (9E10, Santa Cruz Biotechnology) was used to immunoprecipitate equal amounts of each clarified cell lysate. Immunoprecipitations of endogenous proteins from confluent SJSA cells were performed using the following antibodies: mouse IgG (B2, Santa Cruz Biotechnology), anti-Mdm2 (SMP14, Santa Cruz Biotechnology), and anti-RPL36A (43A, Santa Cruz Biotechnology). After an overnight incubation with the antibodies, pre-blocked Protein G Sepharose beads (GE Healthcare) were added to the cell lysates for 1 hour. Unbound proteins were removed by washing four times with an excess of Lysis Buffer.

Ubiquitination and cycloheximide assays

Ubiquitination assays were carried out as described previously (Daftuar et al., 2013). In the case of cycloheximide assays, 100 μg/mL cycloheximide was added to transfected cells for the indicated amounts of time. After immunoblotting, band intensities were quantified using Odyssey software (LI-COR Biosciences), and p53 levels were normalized to actin for each sample. The

half-life of p53 protein was calculated using a one-phase exponential decay model (GraphPad Prism).

Quantitative RT-PCR

Quantitative RT-PCR was carried out as described previously (Daftuar et al., 2013). Primer sequences (Table 3.S2) were validated for efficiency and specificity prior to the start of experimentation.

Immunofluorescent microscopy

Immunofluorescent microscopy was carried out as described previously (Daftuar et al., 2013).

RESULTS

RPL36A interacts with Mdm2

RPL36A and its closely related homolog RPL36AL were identified as potential Mdm2 binding partners in a yeast two-hybrid screen (Zhu et al., 2009). Our first step was to validate this finding in mammalian cells. RPL36A was cloned into a mammalian expression vector, and RPL36AL was sub-cloned using site-directed mutagenesis. When Mdm2 and RPL36A were transfected into the H1299 lung carcinoma cell line, immunoprecipitating RPL36A allowed for the co-immunoprecipitation of Mdm2 (Figure 3.1a). Importantly, H1299 cells are null for p53, indicating RPL36A can interact with Mdm2 independently of p53. On the other hand, the presence of p53 does not interfere with this interaction since a similar result was seen in U2OS cells, an osteosarcoma cell line that contains wildtype p53 (Figure 3.S1).

To further validate this finding, we observed that the endogenous Mdm2 and RPL36A proteins could co-immunoprecipitate in the SJSA osteosarcoma cell line (Figure 3.1b). Specifically, while the Mdm2 antibody used immunoprecipitated Mdm2 and co-immunoprecipitated RPL36A, the RPL36A antibody used did not co-immunoprecipitate Mdm2. This suggests the epitope of the RPL36A antibody may obscure the site of interaction with Mdm2, or that only a small fraction of cellular RPL36A interacts with Mdm2. It should also be noted that since RPL36A and RPL36AL differ by only a single amino acid, the antibody recognizes both proteins. Thus it is unclear if Mdm2 is interacting with RPL36A,

RPL36AL, or (most likely) both proteins. The interaction between the endogenous proteins also persisted in the presence of RNase, indicating their interaction is independent of RNA (data not shown).

To extend the finding that RPL36A and Mdm2 can interact, we mapped the sites of interaction using a panel of Mdm2 deletion mutants that were previously generated (Zhu et al., 2009). Given the highly basic nature of RPL36A, we anticipated the two proteins would most likely interact via the acidic domain of Mdm2, which lies between residues 237 and 288. Indeed, a Mdm2 deletion construct missing residues 222-272 failed to interact with RPL36A, as did a second deletion construct missing residues 222-340 (Figure 3.S2). Many of the ribosomal proteins that have been shown to interact with Mdm2 bind to its acidic domain; the remainder bind to the adjacent Zinc finger region between residues 288 and 331. RPS15 and RPS20 also have secondary binding sites at the N-terminus and C-terminus of Mdm2 (Daftuar et al., 2013) (Figure 3.1c).

RPL36A downregulates p53

We next sought to determine if the physical interaction between RPL36A and Mdm2 has a functional consequence in cells. Mdm2 is most famous as the key negative regulator of p53, so we hypothesized RPL36A would inhibit Mdm2 and increase levels of p53. Using a standard cell-based p53 degradation assay, the introduction of Flag-Mdm2 to U2OS cells that were transfected with HA-p53 led to the downregulation of p53 levels. Unexpectedly, when Myc-RPL36A was

added to the system, levels of p53 dropped even further (Figure 3.2a). This is in contrast to over a dozen other ribosomal proteins that have all been shown to abrogate the Mdm2-mediated degradation of p53 and upregulate p53 levels (Lohrum et al., 2003; Zhang et al., 2003; Dai et al., 2004; Jin et al., 2004; Dai and Lu, 2004; Chen et al., 2007; Zhu et al., 2009; Zhang et al., 2010; Xiong et al., 2011; Sun et al., 2011; Zhou et al., 2013; Zhang et al., 2013; Cui et al., 2013; Daftuar et al., 2013). Indeed, as was previously published, we confirmed that the addition of RPL11 upregulated p53 levels in our assay.

Furthermore, RPL36A was also able to downregulate levels of endogenous p53 in U2OS cells (Figure 3.2b). This novel phenotype is the opposite of what been seen for all the other RPs that have been shown to regulate the Mdm2-p53 axis; when any of them are overexpressed, they increase levels of ectopic and endogenous p53. It is possible that unlike other ectopic RPs, overexpression of RPL36A disrupted global protein synthesis. However, this is unlikely because levels of Myc-RPL36A were not very strongly overexpressed relative to endogenous RPL36A / RPL36AL, yet it was still able to exert this effect. Additionally, immunofluorescence (IF) assays showed Myc-RPL36A localizing primarily to the nucleolus, not to the cytoplasm of H1299 cells (Figure 3.S3), suggesting that Myc-RPL36A, like other ectopic RPs, is being incorporated into newly formed 60S ribosomes and pre-existing 80S ribosomes within the cytoplasm are not perturbed. (The relative absence of signal in the cytoplasm also suggests that, as with other ectopic RPs, incorporation of Myc-

RPL36A into active 80S ribosomes causes it to become inaccessible to detection by antibody.)

In contrast to what was observed with ectopic Myc-RPL36A, levels of p53 increased when either endogenous RPL36A or RPL36AL, or both, were knocked down by siRNA (Figure 3.2c-e). In general, as reviewed in the Introduction (Chapter 1), siRNA directed against other RPs that interact with Mdm2 lead to decreased levels of p53 (the exceptions are siRPL23, siRPL37, siRPS14, siRPS15, siRPS20, and siRPS26, which also upregulate p53 levels). However, siRNA-mediated knockdown of many RPs that do not bind to Mdm2 also lead to an increase in p53 levels. In each of these latter cases, the leading hypothesis is that the removal of an essential RP disrupts ribosomal biogenesis and induces cellular stress that indirectly signals to p53. But ribosomal biogenesis may remain undisrupted when RPL36A is knocked down by sequence-specific siRNA that leaves RPL36AL levels intact (Figure 3.S4), and vice versa, since they can substitute for each other within the ribosome (Uechi et al., 2002; Baouz et al., 2009). Hence, the increase in p53 levels observed upon treatment of cells with siRPL36A or siRPL36AL may be due to a direct signal to the Mdm2-p53 axis. One additional clue that siRPL36A may not disrupt ribosomal biogenesis came from looking at overall levels of proteins within the cell. Coomassie Blue staining showed that siRNA against either RPL36A or RPL36AL did not change global protein levels (Figure 3.S5), although we cannot rule out an effect on scarce short-lived cellular proteins.

RPL36A augments turnover of p53 protein

Our next step was to determine the mechanism by which RPL36A regulates p53 levels. Other ribosomal proteins that interact with Mdm2 have been shown to inhibit Mdm2-mediated degradation of p53 and prolong the half-life of the p53 protein. But unlike other ribosomal proteins, RPL36A enhanced Mdm2-mediated ubiquitination of p53 in a dose-dependent manner (Figure 3.3a). In addition, the proteasome was necessary for knockdown of RPL36A or RPL36AL to affect p53 levels; when its function was inhibited by the addition of MG132, siRPL36A or siRPL36AL were no longer able to increase levels of p53 (Figure 3.3b). In support of this finding, cycloheximide chase assays showed that knockdown of either RPL36A or RPL36AL increased the half-life of p53 by approximately two-fold. Furthermore, knockdown of both of them simultaneously led to an increase in the half-life of p53 in excess of 10-fold, indicating p53 degradation was nearly completely blocked (Figure 3.3c).

RPL36A inhibits the p53 response to ribosomal stress

We next explored if the observed function interaction between RPL36A and the Mdm2-p53 axis has any relevance *in vivo*. Ribosomal stress can be induced by the addition of low doses of actinomycin D (ActD) or 5-fluorouracil (5FU) to cells. ActD is a potent inhibitor of RNA polymerase I (RNA Pol I), the enzyme responsible for transcribing most of the cell's rRNA (5S rRNA is

transcribed by RNA polymerase III). At higher concentrations, ActD can also inhibit Pol II and thus shut down transcription of mRNA. 5FU is a derivative of uracil that can interfere with the synthesis of thymidine and can be incorporated into elongating RNA to cause cell cycle arrest. At higher doses, 5FU can also be incorporated into replicating DNA and cause apoptosis.

Other ribosomal proteins that interact with Mdm2 have been shown to be necessary for a full response to ribosomal stress; when they are knocked down by siRNA, the p53 response is attenuated. On the other hand, when siRNA was directed against RPL36A or RPL36AL and ActD was applied, the p53 response was accentuated (Figure 3.4a-b). Similar results were seen for the response to 5FU (Figure 3.4c-d). Only when RPL36A was overexpressed did the response to ribosomal stress become attenuated (Figure 3.4e-f). Again, this is in contrast to other ribosomal proteins whose siRNA-mediated ablation leads to attenuation of the stress response.

RPL36A and RPL36AL relocalize in response to ribosomal stress

While exploring the mechanism of how RPL36A is able to regulate the Mdm2-p53 axis, we observed that the ectopic Myc-RPL36A protein was quite unstable. It was degraded by the proteasome, and its half-life was quite short (Figure 3.5a-b). However, the endogenous RPL36A / RPL36AL protein was extremely stable, with no detectable degradation following 6 hours of cycloheximide treatment (Figure 3.5c). This makes sense given its status as a

ribosomal protein, whose half-lives are generally measured in many hours or days. Therefore, we were quite surprised to observe an apparent change in the levels of endogenous RPL36A / RPL36AL proteins in response to ribosomal stress (Figure 3.5d). This is in contrast to ribosomal proteins such as RPL11, RPL23, and RPL26, whose levels do not change in response to ribosomal stress (Dai et al., 2004; Zhang et al., 2010).

One recent report found that RPS27A was degraded in response to ActD-induced ribosomal stress (Sun et al., 2011), however we found inhibition of proteasomal degradation did not affect the apparent drop in RPL36A / RPL36AL levels (Figure 3.S6). Additionally, the levels of RPL36A and RPL36AL mRNA did not change in response to ribosomal stress (Figure 3.S7). However, a previous publication on RPL36A reported that it relocalizes to cell surfaces during doxorubicin-induced stress (Nishida et al., 2002), so we explored if a similar phenomenon could be observed during ribosomal stress. We assayed for the levels of RPL36A and RPL36AL levels in both a soluble-only fraction and in complete cell lysate. For the soluble fraction, we followed our standard practice of performing a brief, low-speed spin after lysis to separate out the insoluble membranes and debris prior to gel electrophoresis and immunoblotting. For the complete cell lysate, we omitted this spin and loaded the entire cell lysate onto the gel. As shown in Figure 3.5e, levels of RPL36A and RPL36AL were steady when looking at the complete cell lysate but appeared to drop in the soluble fraction. Additionally, if the insoluble debris was resuspended and loaded onto

the gel, levels of RPL36A and RPL36AL appeared to go up in response to ribosomal stress (Figure 3.5f). We surmise that RPL36A and RPL36AL are re-partitioned between soluble and particular cell fractions in response to ribosomal stress.

DISCUSSION

Over a dozen ribosomal proteins (RPs) have previously been identified as tumor suppressors that activate p53 (Lohrum et al., 2003; Zhang et al., 2003; Dai et al., 2004; Jin et al., 2004; Dai and Lu, 2004; Chen et al., 2007; Zhu et al., 2009; Zhang et al., 2010; Xiong et al., 2011; Sun et al., 2011; Zhou et al., 2013; Zhang et al., 2013; Cui et al., 2013; Daftuar et al., 2013). One of those 12, RPL26, has an additional mechanism for stimulating p53 activity; it can also increase p53 levels by augmenting translation of its mRNA (Takagi et al., 2005; Chen et al., 2010), and several more RPs have been shown to indirectly activate p53 when their ablation by siRNA leads to a disruption to ribosomal biogenesis (Zhou et al., 2012). This led to a hypothesis that RPs could be classified as "effectors" or "detectors" (Llanos and Serrano, 2010; Daftuar et al., 2010). "Effector" RPs interact with Mdm2, increase levels of p53 when overexpressed by inhibiting Mdm2 mediated ubiquitination and degradation, generally decrease levels of p53 when knocked down by siRNA, but are necessary for a full response to actinomycin D-induced ribosomal stress. "Detector" RPs do not interact with Mdm2, do not affect p53 when overexpressed, increase p53 when knocked down by siRNA by causing an impairment to ribosomal biogenesis, and indirectly signal to the Mdm2-p53 axis through the "effector" RPL11.

Here, we present evidence that RPL36A has a novel property as a ribosomal protein that can inhibit p53 rather than activate it. Based upon the previous effector / detector model, we suggest it may be the first RP that can be

considered a “repressor” of the Mdm2-p53 axis. Like “effector” RPs, RPL36A interacts with Mdm2, but unlike them, causes p53 levels to drop when overexpressed by enhancing Mdm2-mediated ubiquitination and degradation. Like “detector” RPs, knockdown of RPL36A increases levels of p53, but unlike either “detectors” or “effectors”, overexpression of RPL36A attenuates the p53 response to ribosomal stress. Perhaps most relevant to what may happen *in vivo*, endogenous RPL36A relocalizes in response to ribosomal stress and this movement may allow for a full p53 response.

RPL36A appears to function through enhancement of Mdm2-mediated degradation, but additional mechanisms for regulating p53 levels are possible. For example, RPL26 has multiple mechanisms for regulating p53 levels. Besides binding to the central acidic region of Mdm2 and inhibiting its E3 ubiquitin ligase activity, it can also bind to both the 5’ and 3’ UTR of p53 mRNA and stimulate its translation in response to irradiation (Takagi et al., 2005; Chen et al., 2010).

Perhaps it should not be too surprising that not all RPs affect the Mdm2-p53 axis the same way. In the case of Myc, a transcription factor that can lead to increased cellular proliferation, various RPs have been shown to affect its activity in different ways. For example, RPL11 and RPS14 can bind to and inhibit Myc from transactivation target genes (Dai et al., 2007; Dai et al., 2010; Zhou et al., 2013), while RPL23 can indirectly promote Myc activation by inhibiting its antagonist Miz1 (Wanzel et al., 2008). RPL11 was also shown to inhibit Myc in response to ribosomal stress by targeting its mRNA for degradation via miR24

(Challagundla et al., 2011). It remains to be seen if other RPs can also function as “repressors” of p53 like RPL36A.

In cells, RPs are made in excess of the amount needed for ribosomal biogenesis (Warner and McIntosh, 2009), thus many RPs are continually degraded (Lam et al., 2007). If their degradation is inhibited, they may be able to translocate between the nucleolus and the nucleoplasm (Chen and Huang, 2001). In that case, perhaps the “effector” subset of cellular RPs would inappropriately signal to p53, and RPL36A is needed to prevent this. Additionally, a few studies indicate excess RPL5 and RPL11 may be protected from degradation by pre-assembling with 5S rRNA in the nucleolus (Steitz et al., 1988; Zhang et al., 2007). While their preservation may serve important functions with respect to ribosome assembly, it has been a mystery why they do not signal to Mdm2 and lead to p53 activation in the absence of cellular stress. Our data may be the first clue in solving this mystery; RPL36A may serve as a barrier to inappropriate signaling to Mdm2 by RPL5 and RPL11 in the absence of ribosomal stress. Finally, p53 activity must be tightly controlled even in the presence of stress in order to prevent its theoretical pro-survival effects from taking over (Vousden and Prives, 2009), and perhaps RPL36A also plays a role in regulating p53 activity with respect to ribosomal or nucleolar stress. As more RPs are explored in future studies, the model of “effector”, “detector” and “repressor” RPs may need further refinement.

REFERENCES

Amsterdam, A., Sadler, K.C., Lai, K., Farrington, S., Bronson, R.T., Lees, J.A., and Hopkins, N. (2004). Many ribosomal protein genes are cancer genes in zebrafish. PLoS biology *2*, E139.

Baouz, S., Woisard, A., Sinapah, S., Le Caer, J.P., Argentini, M., Bulygin, K., Aguié, G., and Hountondji, C. (2009). The human large subunit ribosomal protein L36A-like contacts the CCA end of P-site bound tRNA. Biochimie *91*, 1420-1425.

Bevort, M, and Leffers, H. (2000). Down regulation of ribosomal protein mRNAs during neuronal differentiation of human NTERA2 cells. Differentiation *66*, 81-92.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J., and Lamond, A.I. (2007). The multifunctional nucleolus. Nature reviews. Molecular cell biology *8*, 574-585.

Challagundla, K.B., Sun, X.X., Zhang, X., DeVine, T., Zhang, Q., Sears, R.C., and Dai, M.S. (2011). Ribosomal protein L11 recruits miR-24/miRISC to repress c-Myc expression in response to ribosomal stress. Molecular and cellular biology *31*, 4007-4021.

Chen, D., and Huang, S. (2001). Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. The Journal of cell biology *153*, 169-176

Chen, D., Zhang, Z., Li, M., Wang, W., Li, Y., Rayburn, E.R., Hill, D.L., Wang, H., and Zhang, R. (2007). Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene *26*, 5029-5037.

Chen, J., and Kastan, M.B. (2010). 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes & development *24*, 2146-2156.

Cui, D., Li, L., Lou, H., Sun, H., Ngai, S.M., Shao, G., and Tang, J. (2013). The ribosomal protein S26 regulates p53 activity in response to DNA damage. Oncogene.

Daftuar, L., Zhu, Y., Jacq, X., and Prives, C. (2013). Ribosomal proteins RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network. PLoS one.

Daftuar, L., Zhu, Y., and Prives, C. (2010). Ribosomal protein L37 and the p53 network. Cell cycle *9*, 4259.

Dai, M.S., Arnold, H., Sun, X.X., Sears, R., and Lu, H. (2007). Inhibition of c-Myc activity by ribosomal protein L11. The EMBO journal *26*, 3332-3345.

Dai, M.S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. The Journal of biological chemistry *279*, 44475-44482.

Dai, M.S., Sun, X.X., and Lu, H. (2010). Ribosomal protein L11 associates with c-Myc at 5 S rRNA and tRNA genes and regulates their expression. The Journal of biological chemistry *285*, 12587-12594.

Dai, M.S., Zeng, S.X., Jin, Y., Sun, X.X., David, L., and Lu, H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Molecular and cellular biology *24*, 7654-7668.

Deisenroth, C. and Zhang, Y. (2010). Ribosome biogenesis surveillance: probing the ribosomal protein-Mdm2-p53 pathway. Oncogene *29*, 4253-4260.

Fabijanski, S. and Pellegrini, M. (1981). Identification of proteins at the peptidyl-tRNA binding site of rat liver ribosomes. Mol Gen Genet. *184*, 551-556.

Gomez-Lazaro, M., Fernandez-Gomez, F.J., and Jordan, J. (2004). p53: twenty five years understanding the mechanism of genome protection. Journal of physiology and biochemistry *60*, 287-307.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS letters *420*, 25-27.

Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Molecular and cellular biology *24*, 7669-7680.

Kappeler, K.V., Zhang, J., Dinh, T.N., Strom, J.G., and Chen, Q.M. (2012). Histone deacetylase 6 associates with ribosomes and regulates de novo protein translation during arsenite stress. Toxicol Sci. *127*, 246-55.

Kim, J.H., You, K.R., Ki, I.H., Cho, B.H., Kim, C. Y., and Kim, D.G. (2004). Over-expression of the ribosomal protein L36a gene is associated with cellular proliferation in hepatocellular carcinoma. Hepatology. *39*, 129-138.

Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Lam, Y.W., Lamond, A.I., Mann, M., and Andersen, J.S. (2007). Analysis of nucleolar protein dynamics reveals the nuclear degradation of ribosomal proteins. Current biology : CB *17*, 749-760.

Levine, A.J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nature reviews. Cancer *9*, 749-758.

Llanos, S., and Serrano, M. (2010). Depletion of ribosomal protein L37 occurs in response to DNA damage and activates p53 through the L11/MDM2 pathway. Cell cycle *9*, 4005-4012.

Lohrum, M.A., Ludwig, R.L., Kubbutat, M.H., Hanlon, M., and Vousden, K.H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer cell *3*, 577-587.

Marion, M.J. and Marion, C. (1987). Localization of ribosomal proteins on the surface of mammalian 60S ribosomal subunits by means of immobilized enzymes. Correlation with chemical cross-linking data. Biochem Biophys Res Commun. *149*, 1077-1083.

Naora, H., Takai, I., Adachi, M., and Naora, H. (1998). Altered cellular responses by varying expression of a ribosomal protein gene: sequential coordination of enhancement and suppression of ribosomal protein S3a gene expression induces apoptosis. The Journal of cell biology *141*, 741-753.

Nishida, J., Shiratsuchi, A., Nadano, D., Sato, T.A., and Nakanishi, Y. (2002). Structural change of ribosomes during apoptosis: degradation and externalization of ribosomal proteins in doxorubicin-treated Jurkat cells. J Biochem. *131*, 485-493.

Peart, M.J., Poyurovsky, M.V., Kass, E.M., Urist, M., Verschuren, E.W., Summers, M.K., Jackson, P.K., and Prives C. (2010). APC/C(Cdc20) targets E2F1 for degradation in prometaphase. Cell Cycle. *9*, 3956-64.

Steitz, J.A., Berg, C., Hendrick, J.P., La Branche-Chabot, H., Metspalu, A., Rinke, J., and Yario, T. (1988). A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. J Cell Biol. *106*, 545-556.

Sun, X.X., DeVine, T., Challagundla, K.B., and Dai, M.S. (2011). Interplay between ribosomal protein S27a and MDM2 protein in p53 activation in response to ribosomal stress. The Journal of biological chemistry *286*, 22730-22741.

Takagi, M., Absalon, M.J., McLure, K.G., and Kastan, M.B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell *123*, 49-63.

Uechi, T., Maeda, N., Tanaka, T., and Kenmochi, N. (2002). Functional second genes generated by retrotransposition of the X-linked ribosomal protein genes. Nucleic Acids Res. *30*, 5369-5375.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Wanzel, M., Russ, A.C., Kleine-Kohlbrecher, D., Colombo, E., Pelicci, P.G., and Eilers, M. (2008). A ribosomal protein L23-nucleophosmin circuit coordinates Mizl function with cell growth. Nature cell biology *10*, 1051-1061.

Warner, J.R., and McIntosh, K.B. (2009). How common are extraribosomal functions of ribosomal proteins? Molecular cell *34*, 3-11.

Wesseling, S., Essers, P.B., Koeners, M.P., Pereboom, T.C., Braam, B., van Faassen, E.E., Macinnes, A.W., and Joles, J.A. (2011). Perinatal exogenous nitric oxide in fawn-hooded hypertensive rats reduces renal ribosomal biogenesis in early life. Front Genet.

Xiong, X., Zhao, Y., He, H., and Sun, Y. (2011). Ribosomal protein S27-like and S27 interplay with p53-MDM2 axis as a target, a substrate and a regulator. Oncogene *30*, 1798-1811.

Zhang, J., Harnpicharnchai, P., Jakovljevic, J., Tang, L., Guo, Y., Oeffinger, M., Rout, M.P., Hiley, S.L., Hughes, T., and Woolford, J.L. Jr. (2007). Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes. Genes Dev. *21*, 2580-2592.

Zhang, X., Wang, W., Wang, H., Wang, M.H., Xu, W., and Zhang, R. (2013). Identification of ribosomal protein S25 (RPS25)-MDM2-p53 regulatory feedback loop. Oncogene *32*, 2782-2791.

Zhang, Y., Wang, J., Yuan, Y., Zhang, W., Guan, W., Wu, Z., Jin, C., Chen, H., Zhang, L., Yang, X*., et al.* (2010). Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26. Nucleic acids research *38*, 6544-6554.

Zhang, Y., Wolf, G.W., Bhat, K., Jin, A., Allio, T., Burkhart, W.A., and Xiong, Y. (2003). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and

mediates a p53-dependent ribosomal-stress checkpoint pathway. Molecular and cellular biology *23*, 8902-8912.

Zhou, X., Hao, Q., Liao, J., Zhang, Q., and Lu, H. (2013a). Ribosomal protein S14 unties the MDM2-p53 loop upon ribosomal stress. Oncogene *32*, 388-396.

Zhou, X., Hao, Q., Liao, J.M., Liao, P., and Lu, H. (2013b). Ribosomal protein S14 negatively regulates c-Myc activity. The Journal of biological chemistry.

Zhou, X., Liao, J.M., Liao, W.J., and Lu, H. (2012). Scission of the p53-MDM2 Loop by Ribosomal Proteins. Genes & cancer *3*, 298-310.

Zhu, Y., Poyurovsky, M.V., Li, Y., Biderman, L., Stahl, J., Jacq, X., and Prives, C. (2009). Ribosomal protein S7 is both a regulator and a substrate of MDM2. Molecular cell *35*, 316-326.

FIGURE LEGENDS

Figure 3.1. RPL36A interacts with Mdm2. (a) Association of ectopically expressed RPL36A and Mdm2. H1299 cells were transfected with Flag-Mdm2 (1.2 μg), Myc-RPL36A (1.2 μg), or both. (0.1 μg GFP was added as a control for transfection efficiency.) Cell lysates were subjected to immunoprecipitation (IP) and immunoblotting as described. **(b) Association of endogenously expressed RPL36A and Mdm2.** SJSA cell lysates were subjected to IP with α-Mdm2, and co-IP of endogenous RPL36A was detected by immunoblot. **(c) Schematic of sites of interaction between RPs and Mdm2.** In the upper portion, Mdm2 protein landmarks are depicted; in the bottom portion, the regions of Mdm2 bound by RPL36A and other published RPs are depicted.

Figure 3.2. RPL36A decreases levels of p53 protein. (a) RPL36A enhances degradation of ectopic HA-p53. U2OS cells were transfected with Flag-Mdm2 (1.2 μg), HA-p53 (0.3 μg), and Myc-RPs (1.0 μg). Ectopic Mdm2, p53, and RP levels were detected by immunoblotting with α-Flag, α-HA, and α-Myc. **(b) Decrease in levels of endogenously expressed p53 by RPL36A.** U2OS cells were transfected with increasing amounts of Myc-RPL36A (0-3.0 μg). **(c-e) Knockdown of RPL36A or RPL36AL increases levels of p53.** SJSA cells were transfected with siRNA targeting RPL36A or RPL36AL (0 nM, 50 nM, 100 nM, 200 nM) or both (100nM or 50 nM + 50 nM in panel (e)). Cells were split into fractions for immunoblot and quantitative RT-PCR (qRT-PCR). Cell lysates were

subjected to immunoblotting with the indicated antibodies and band intensities were quantified and normalized to actin. Note the RPL36A antibody cannot distinguish between RPL36A and RPL36AL, so a double knockdown of both is needed to see an effect on the protein. The relative expression of RPL36A or RPL36AL mRNA was determined in triplicate and normalized to GAPDH.

Figure 3.3. RPL36A destabilizes p53 protein. (a) Enhancement of Mdm2-mediated ubiquitination of p53 by RPL36A. H1299 were seeded in 60mM tissue culture plates and transfected with HA-Ubiquitin (3.0 μg), p53 (0.75 μg), Flag-Mdm2 (7.5 μg), and Myc-RPL36A (9.0-13.5 μg). 25 μM MG132 was added for 6 hours, and ubiquitinated p53 species were assayed by immunoprecipitating with α-p53 and immunoblotting with α-HA. Inputs and IPs were run on separate gels. **(b) RPL36A and RPL36AL affect p53 levels by proteasomal degradation.** U2OS cells were transfected with 100 nM siRNA targeting RPL36A, RPL36AL, or both as indicated. 30 μM MG132 was added for 6 hours, and cells were split into fractions for immunoblot and quantitative RT-PCR. Lysates were subjected to immunoblotting with the indicated antibodies and the relative expression of p21 mRNA was determined in triplicate and normalized to GAPDH.. **(c) RPL36A and RPL36AL affect p53 protein half-life.** U2OS cells were transfected with 100 nM siRNA, and approximately 71 hours after the initial transfection, 100 μg/mL cycloheximide was added to the culture medium. Cells

were harvested at the indicated timepoints and cell lysates were subjected to immunoblotting with the indicated antibodies.

Figure 3.4. RPL36A inhibits the p53 response to ribosomal stress. (a-b) siRNA against RPL36A and RPL36AL augments the response to actinomycin D (ActD). U2OS cells were transfected with 100 nM siRNA, and approximately 66 hours after the initial transfection, 5nM ActD was added to the culture medium for 6 hours. **(c-d) siRNA against RPL36A and RPL36AL augments the response to 5-fluorouracil (5FU).** SJSA cells were transfected with 100 nM siRNA, and approximately 66 hours after the initial transfection, 50 μM 5FU was added to the culture medium for 6 hours. **(e-f) Ectopic RPL36A inhibits the p53 response to both ActD and 5FU.** U2OS cells were transfected with empty vector (3.0 μg) or Myc-RPL36A (3.0 μg). Approximately 18 hours after the initial transfection, 5nM ActD or 50 μM 5FU was added to the culture medium for 6 hours. Cells were harvested at the indicated timepoints and split into fractions for immunoblot and quantitative RT-PCR (qRT-PCR). Cell lysates were subjected to immunoblotting with the indicated antibodies, and the relative expression of p21 mRNA was determined in triplicate and normalized to GAPDH.

Figure 3.5. RPL36A and RPL36AL relocalize in response to ribosomal stress. (a-b) Ectopic Myc-RPL36A is unstable. U2OS cells were transfected with empty vector (3.0 μg) or Myc-RPL36A (1.0 - 3.0 μg). In (a), 25 μM MG132

was added to the culture medium for 6 hours; in (b) 100 μg/mL cycloheximide (CHX) was added to the culture medium for the indicated amounts of time. **(c) Endogenous RPL36A and RPL36AL are stable in the absence of stress.** 100 μg/mL CHX was added U2OS cells for the indicated amounts of time. **(d-f) Endogenous RPL36AL and RPL36AL relocalize in the presence of ribosomal stress.** 5 nM actinomycin D (ActD) or 50 μM 5-fluorouracil (5FU) were added U2OS cells (in (d) and (e)) or SJSA cells (in (f)) for the indicated amounts of time. In (d), the normal procedure was followed and cell lysates were cleared by centrifuging at 4,000 rpm for 10 minutes prior to gel electrophoresis; the resulting insoluble pellet was discarded. In (e), this spin was skipped and the complete cell lysate was loaded onto the gel. In (f), this spin was performed but the insoluble pellet was saved, resuspended in lysis buffer, and loaded onto the gel. All the immunoblots in panel (f) are from the same gel.

SUPPLEMENTAL FIGURE LEGENDS

Figure 3.S1. RPL36A interacts with Mdm2 in U2OS cells. U2OS cells were transfected with Flag-Mdm2 (1.2 μg), Myc-RPL36A (1.2 μg), or both. (0.1 μg GFP was added as a control for transfection efficiency.) 25 μM MG132 for 6 hours prior to harvesting, and cell lysates were subjected to immmunoprecipitation (IP) and immunoblotting as described.

Figure 3.S2. RPL36A interacts with the central region of Mdm2. H1299 cells were transfected with Myc-RPL36A (1.2 μg), Flag-Mdm2 full length (1.2 μg), Flag-Mdm2 truncation 1-154 (1.2 μg), Flag-Mdm2 truncation 1-220 (0.02 μg), Flag-Mdm2 deletion 222-272 (3.0 μg), Flag-Mdm2 deletion 222-340 (0.5 μg), Flag-Mdm2 deletion 340-437 (0.1 μg), and Flag-Mdm2 truncation 436-482 (0.2 μg). Myc-RPL36A was immunoprecipitated with α-Myc, and co-IP for each Mdm2 construct was assayed by immunoblot. Inputs and IPs were run on separate gels.

Figure 3.S3. RPL36A localizes to nucleoli. H1299 cells were grown on coverslips in 35mM tissue culture plates and transfected with Flag-Mdm2 (1.2 μg), Myc-RPL366A (1.2 μg), or both. Immunofluorescent staining was carried out as described.

Figure 3.S4. siRNAs against RPL36A and RPL36AL are specific. SJSA cells were transfected with 100 nM siRNA targeting RPL36A, RPL36AL, or both as

indicated. Relative expression of each gene was determined in triplicate by quantitative RT-PCR (qRT-PCR) and normalized to GAPDH. (The DNA sequences of RPL36A and RPL36AL differ by 32 nucleotides, so specific siRNA and qRT-PCR primers can be designed.) The average of 3 experiments is plotted, and no significant changes in RPL36A mRNA levels by siRPL36AL were observed using student's t-test. The reverse was also true.

Figure 3.S5. siRNAs against RPL36A and RPL36AL do not change cellular protein levels. U2OS cells were transfected with 100 nM siRNA targeting RPL36A, RPL36AL, or both as indicated. Equal amounts of cell lysates were separated by SDS-PAGE and stained with Coomassie Blue.

Figure 3.S6. Proteasomal inhibition does not stop RPL36A or RPL36AL response to ribosomal stress. 5 nM actinomycin D (ActD) or 50 μM 5-fluorouracil (5FU) were added U2OS cells for 20 hours. At that point, fresh solutions of ActD or 5FU combined with 25 μM MG132 were added to the cells for 4 hours.

Figure 3.S7. RPL36A and RPL36AL mRNAs do not change in response to ribosomal stress. 5 nM actinomycin D (ActD) or 50 μM 5-fluorouracil (5FU) were added U2OS cells for the indicated amounts of time. Relative expression of

each gene was determined in triplicate by quantitative RT-PCR (qRT-PCR) and normalized to GAPDH. (The average of 2 independent experiments is plotted.)

Table 3.T1. qRT-PCR sequences. The primer sequences for the qRT-PCR reactions performed are provided.

FIGURE 3.1

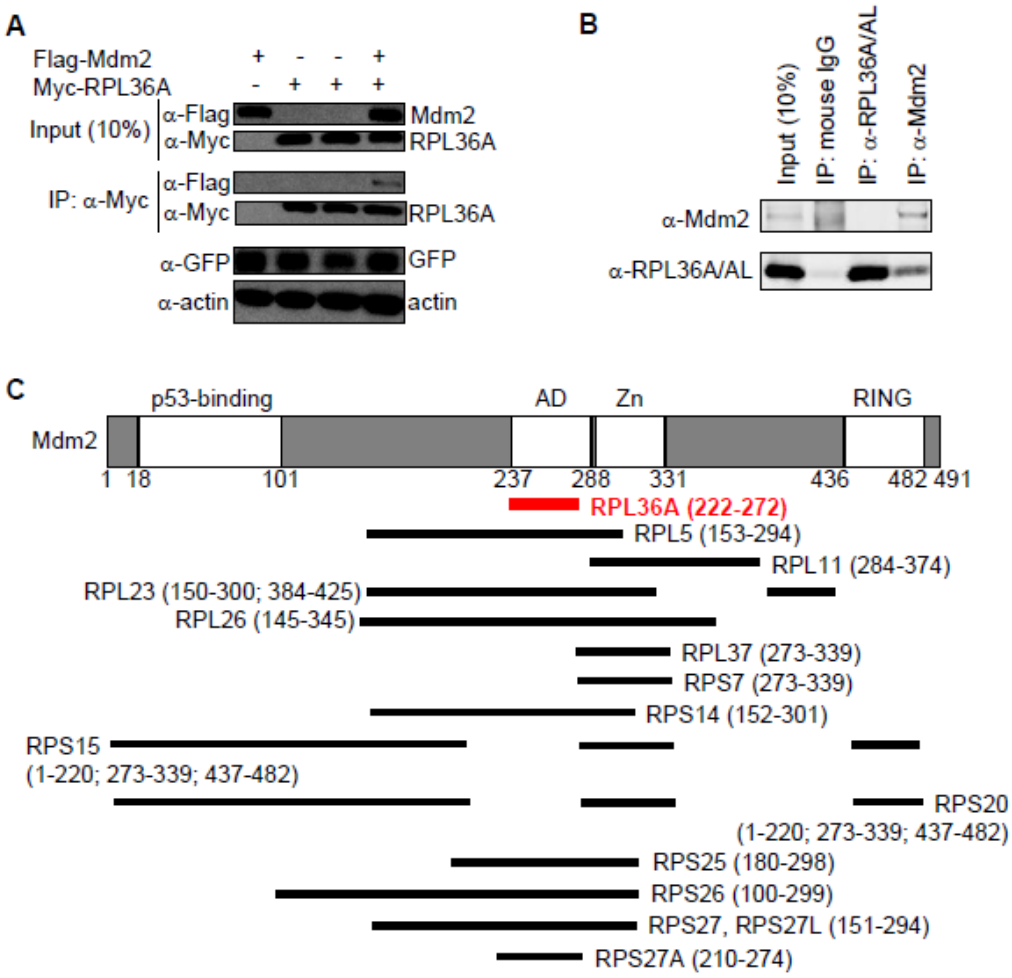

A
Flag-Mdm2 + - - +
Myc-RPL36A - + + +
Input (10%)
α-Flag
α-Myc
Mdm2
RPL36A
IP: α-Myc
α-Flag
α-Myc
RPL36A
α-GFP
GFP
α-actin
actin
B
Input (10%)
IP: mouse IgG
IP: α-RPL36A/AL
IP: α-Mdm2
α-Mdm2
α-RPL36A/AL
C
p53-binding
AD
Zn
RING
Mdm2
1 18 101 237 288 331 436 482 491
RPL36A (222-272)
RPL5 (153-294)
RPL11 (284-374)
RPL23 (150-300; 384-425)
RPL26 (145-345)
RPL37 (273-339)
RPS7 (273-339)
RPS14 (152-301)
RPS15
(1-220; 273-339; 437-482)
RPS20
(1-220; 273-339; 437-482)
RPS25 (180-298)
RPS26 (100-299)
RPS27, RPS27L (151-294)
RPS27A (210-274)

FIGURE 3.2

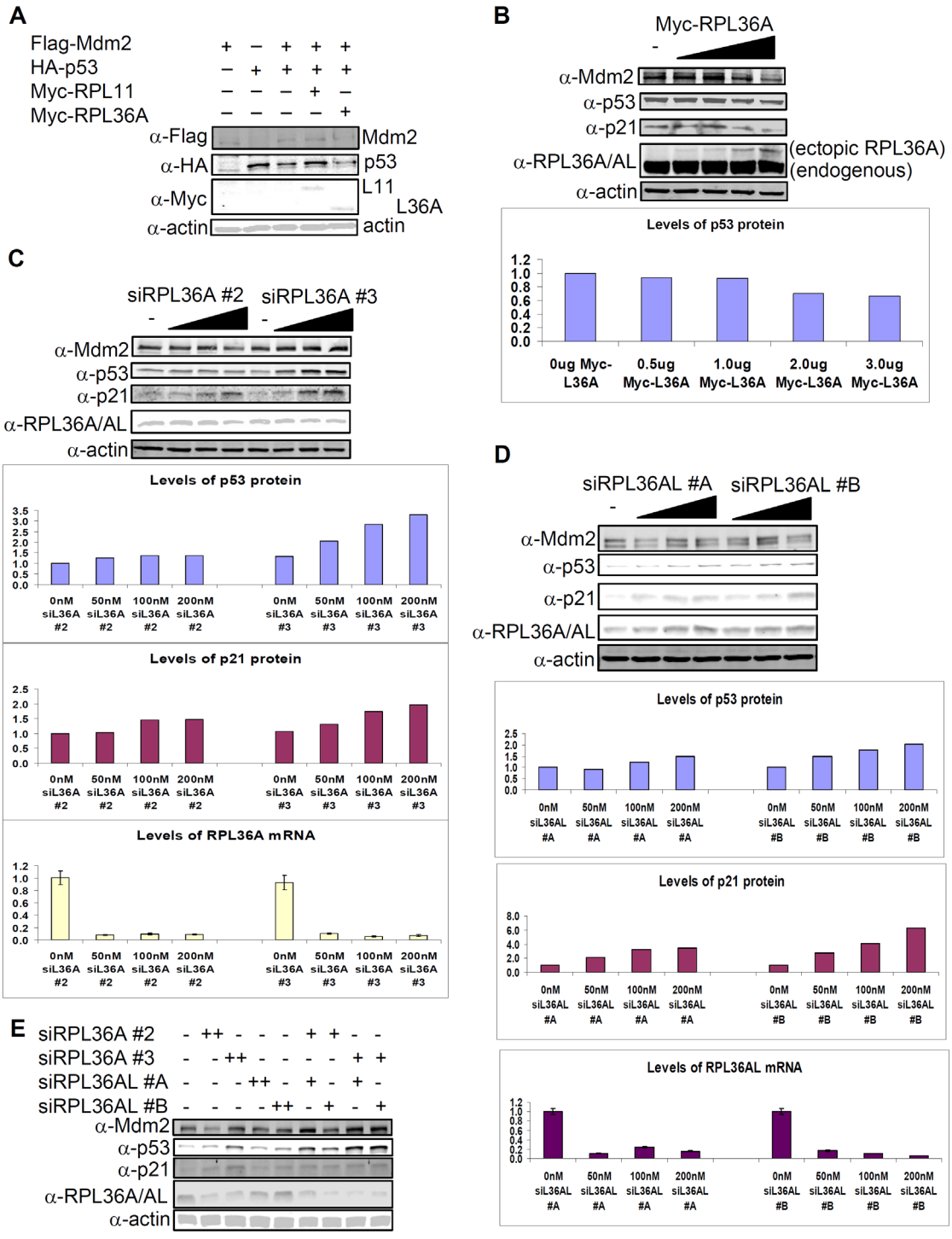

A
Flag-Mdm2
HA-p53
Myc-RPL11
Myc-RPL36A
α-Flag
α-HA
α-Myc
α-actin
Mdm2
p53
L11
L36A
actin
B
Myc-RPL36A
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin
(ectopic RPL36A)
(endogenous)
Levels of p53 protein
0ug Myc-L36A
0.5ug Myc-L36A
1.0ug Myc-L36A
2.0ug Myc-L36A
3.0ug Myc-L36A
C
siRPL36A #2
siRPL36A #3
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin
Levels of p53 protein
Levels of p21 protein
Levels of RPL36A mRNA
0nM siL36A #2
50nM siL36A #2
100nM siL36A #2
200nM siL36A #2
0nM siL36A #3
50nM siL36A #3
100nM siL36A #3
200nM siL36A #3
D
siRPL36AL #A
siRPL36AL #B
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin
Levels of p53 protein
Levels of p21 protein
Levels of RPL36AL mRNA
0nM siL36AL #A
50nM siL36AL #A
100nM siL36AL #A
200nM siL36AL #A
0nM siL36AL #B
50nM siL36AL #B
100nM siL36AL #B
200nM siL36AL #B
E
siRPL36A #2
siRPL36A #3
siRPL36AL #A
siRPL36AL #B
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin

FIGURE 3.3

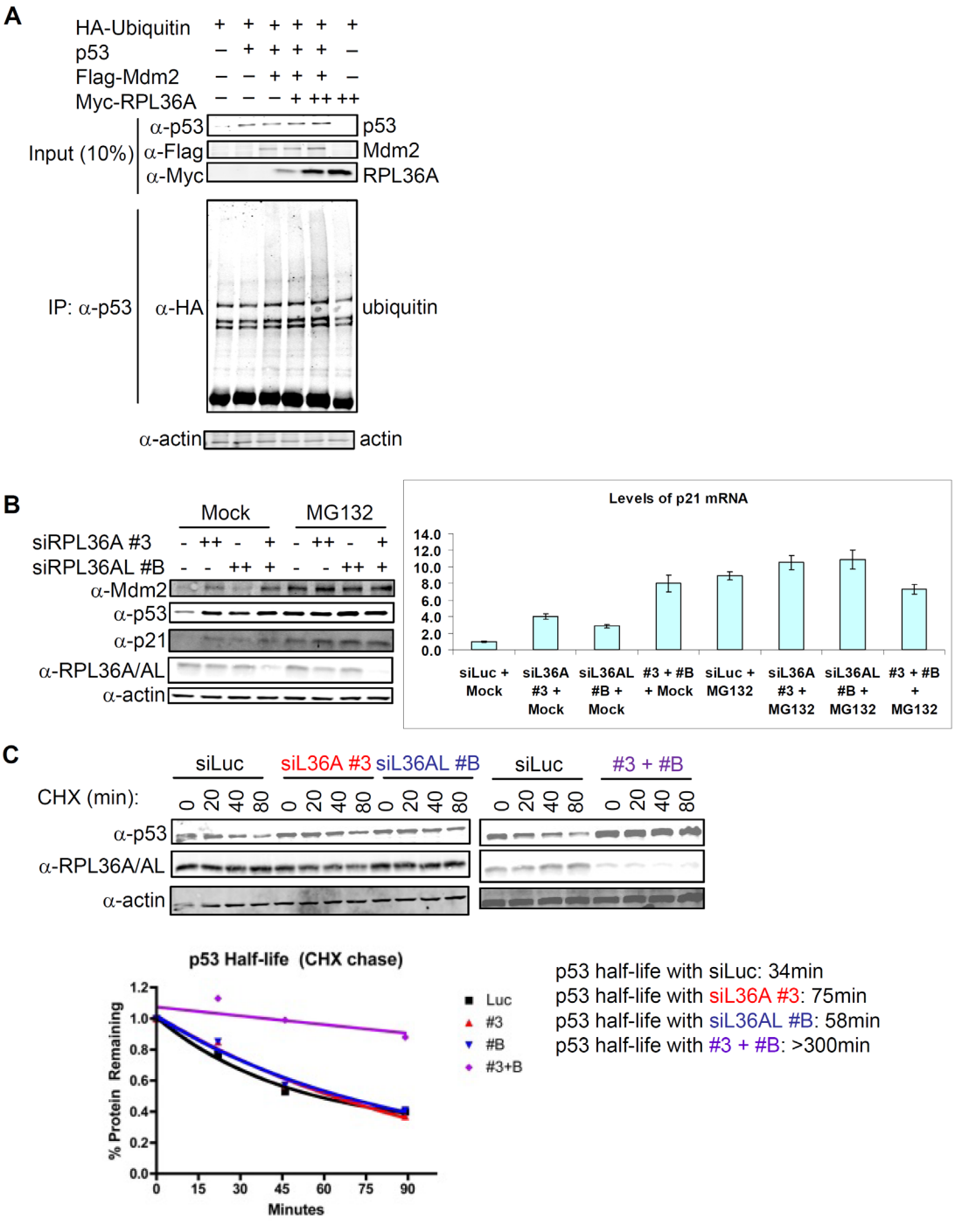

A
HA-Ubiquitin + + + + + +
p53 – + + + + –
Flag-Mdm2 – – + + + –
Myc-RPL36A – – – + ++ ++
Input (10%)
α-p53
p53
α-Flag
Mdm2
α-Myc
RPL36A
IP: α-p53
α-HA
ubiquitin
α-actin
actin
B
Mock
MG132
siRPL36A #3 - ++ - + - ++ - +
siRPL36AL #B - - ++ + - - ++ +
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin
Levels of p21 mRNA
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
siLuc + Mock
siL36A #3 + Mock
siL36AL #B + Mock
#3 + #B + Mock
siLuc + MG132
siL36A #3 + MG132
siL36AL #B + MG132
#3 + #B + MG132
C
siLuc
siL36A #3
siL36AL #B
siLuc
#3 + #B
CHX (min): 0 20 40 80 0 20 40 80 0 20 40 80 0 20 40 80 0 20 40 80
α-p53
α-RPL36A/AL
α-actin
p53 Half-life (CHX chase)
% Protein Remaining
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 15 30 45 60 75 90
Minutes
Luc
#3
#B
#3+B
p53 half-life with siLuc: 34min
p53 half-life with siL36A #3: 75min
p53 half-life with siL36AL #B: 58min
p53 half-life with #3 + #B: >300min

FIGURE 3.4

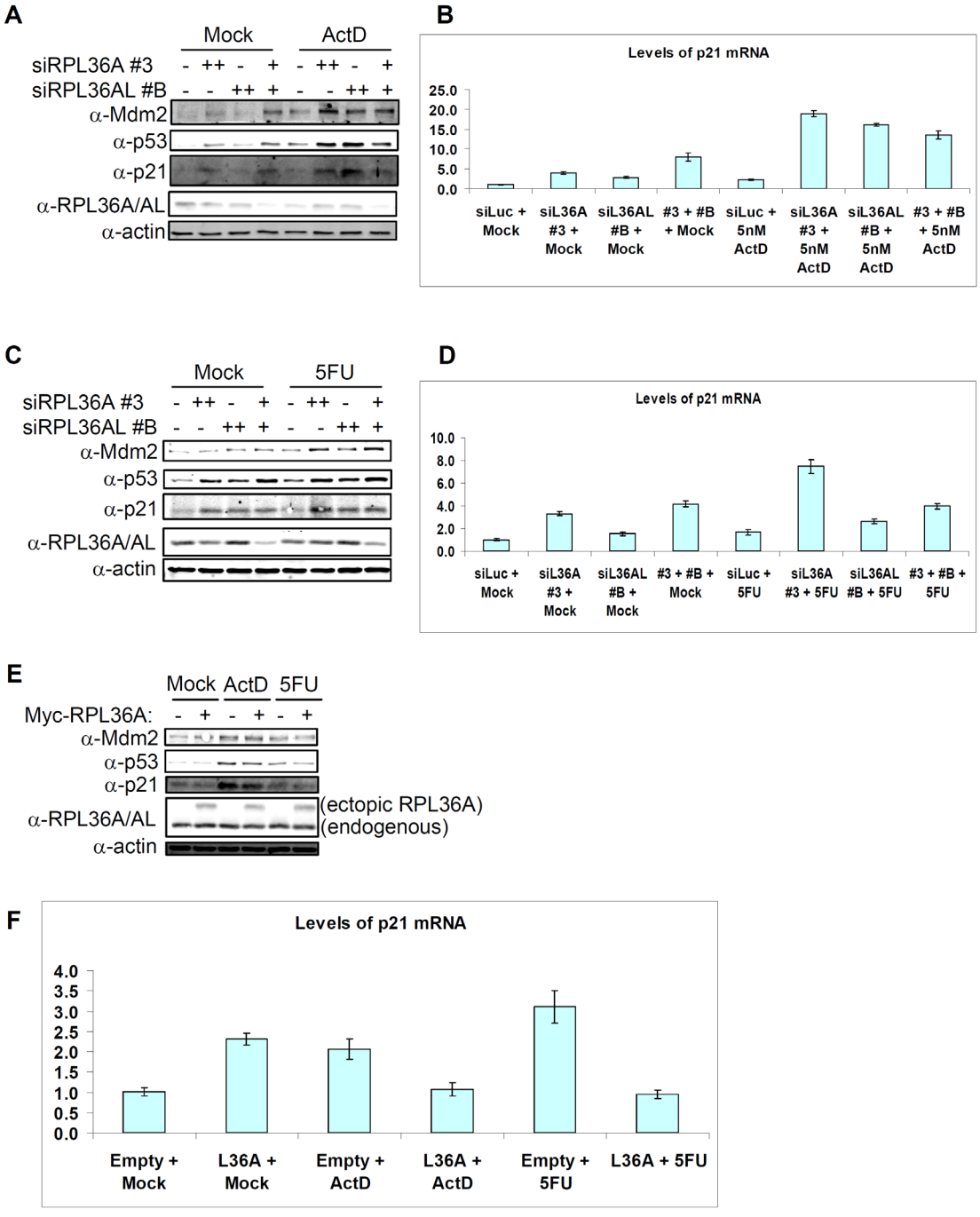

A
Mock
ActD
siRPL36A #3 - ++ - + - ++ - +
siRPL36AL #B - - ++ + - - ++ +
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin
B
Levels of p21 mRNA
25.0
20.0
15.0
10.0
5.0
0.0
siLuc + Mock
siL36A #3 + Mock
siL36AL #B + Mock
#3 + #B + Mock
siLuc + 5nM ActD
siL36A #3 + 5nM ActD
siL36AL #B + 5nM ActD
#3 + #B + 5nM ActD
C
Mock
5FU
siRPL36A #3 - ++ - + - ++ - +
siRPL36AL #B - - ++ + - - ++ +
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin
D
Levels of p21 mRNA
10.0
8.0
6.0
4.0
2.0
0.0
siLuc + Mock
siL36A #3 + Mock
siL36AL #B + Mock
#3 + #B + Mock
siLuc + 5FU
siL36A #3 + 5FU
siL36AL #B + 5FU
#3 + #B + 5FU
E
Mock ActD 5FU
Myc-RPL36A: - + - + - +
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
(ectopic RPL36A)
(endogenous)
α-actin
F
Levels of p21 mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Empty + Mock
L36A + Mock
Empty + ActD
L36A + ActD
Empty + 5FU
L36A + 5FU

FIGURE 3.5

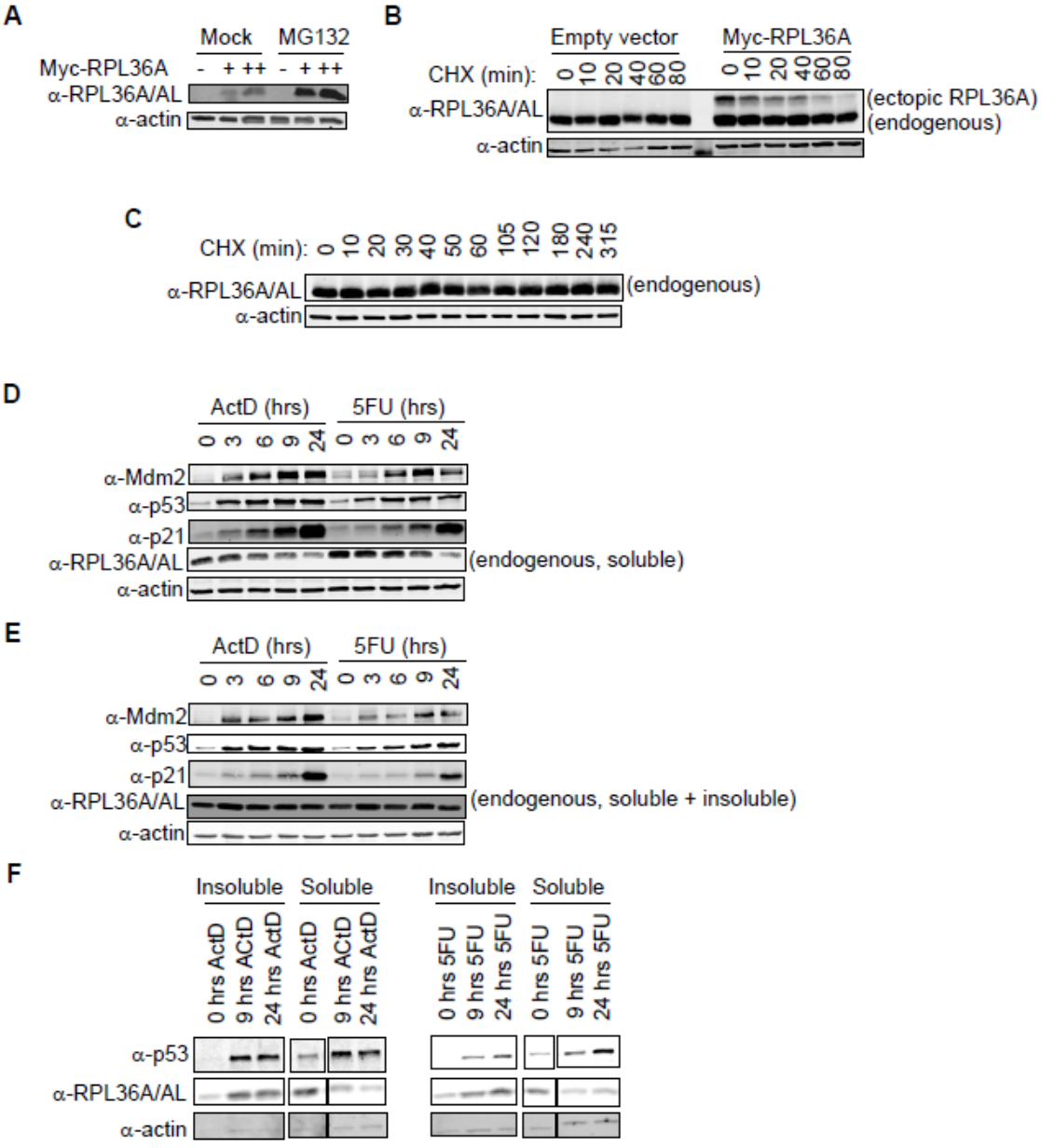

A
Mock
MG132
Myc-RPL36A - + ++ - + ++
α-RPL36A/AL
α-actin
B
Empty vector
Myc-RPL36A
CHX (min): 0 10 20 40 60 80 0 10 20 40 60 80
α-RPL36A/AL
(ectopic RPL36A)
(endogenous)
α-actin
C
CHX (min): 0 10 20 30 40 50 60 105 120 180 240 315
α-RPL36A/AL
(endogenous)
α-actin
D
ActD (hrs)
5FU (hrs)
0 3 6 9 24 0 3 6 9 24
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
(endogenous, soluble)
α-actin
E
ActD (hrs)
5FU (hrs)
0 3 6 9 24 0 3 6 9 24
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
(endogenous, soluble + insoluble)
α-actin
F
Insoluble
Soluble
Insoluble
Soluble
0 hrs ActD
9 hrs ACtD
24 hrs ActD
0 hrs ActD
9 hrs ACtD
24 hrs ActD
0 hrs 5FU
9 hrs 5FU
24 hrs 5FU
0 hrs 5FU
9 hrs 5FU
24 hrs 5FU
α-p53
α-RPL36A/AL
α-actin

FIGURE 3.S1

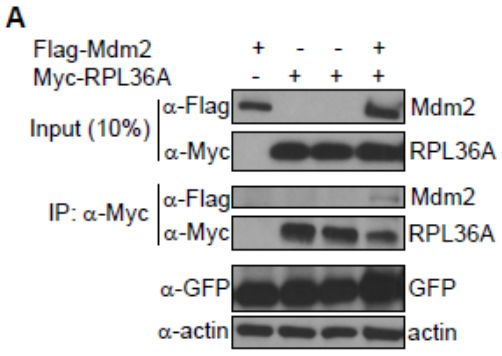

A
Flag-Mdm2 + - - +
Myc-RPL36A - + + +
Input (10%)
α-Flag
Mdm2
α-Myc
RPL36A
IP: α-Myc
α-Flag
Mdm2
α-Myc
RPL36A
α-GFP
GFP
α-actin
actin

FIGURE 3.S2

A

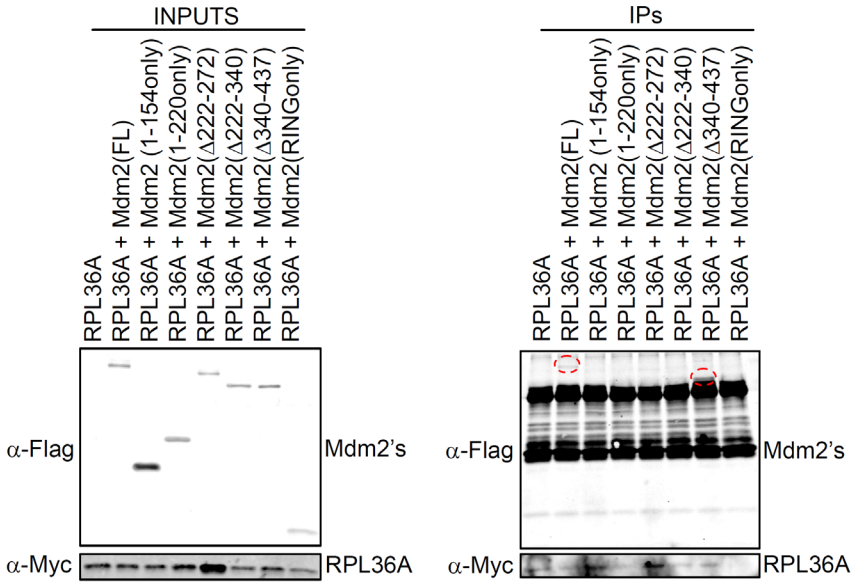

INPUTS
IPs
RPL36A
RPL36A + Mdm2(FL)
RPL36A + Mdm2 (1-154only)
RPL36A + Mdm2(1-220only)
RPL36A + Mdm2(Δ222-272)
RPL36A + Mdm2(Δ222-340)
RPL36A + Mdm2(Δ340-437)
RPL36A + Mdm2(RINGonly)
α-Flag
Mdm2's
α-Myc
RPL36A

FIGURE 3.S3

A

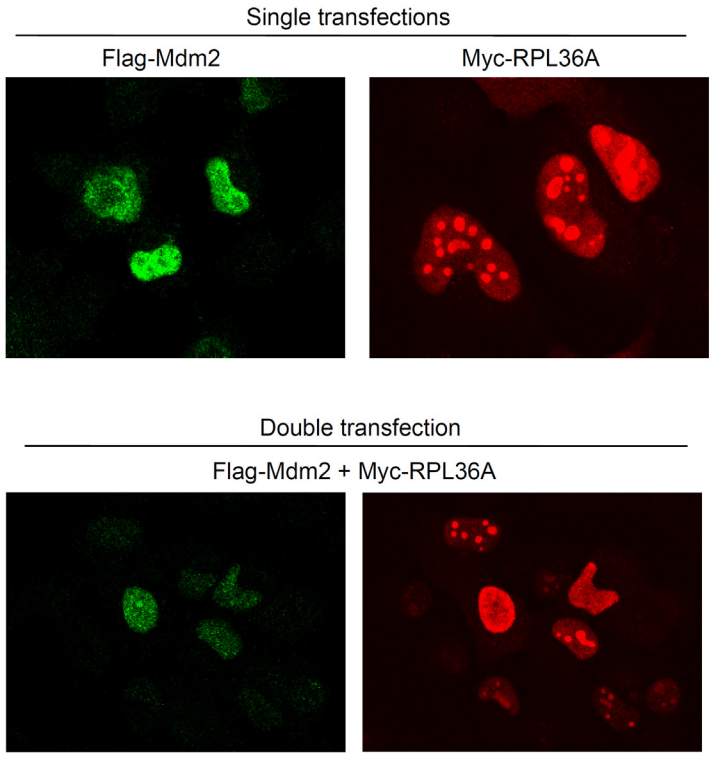
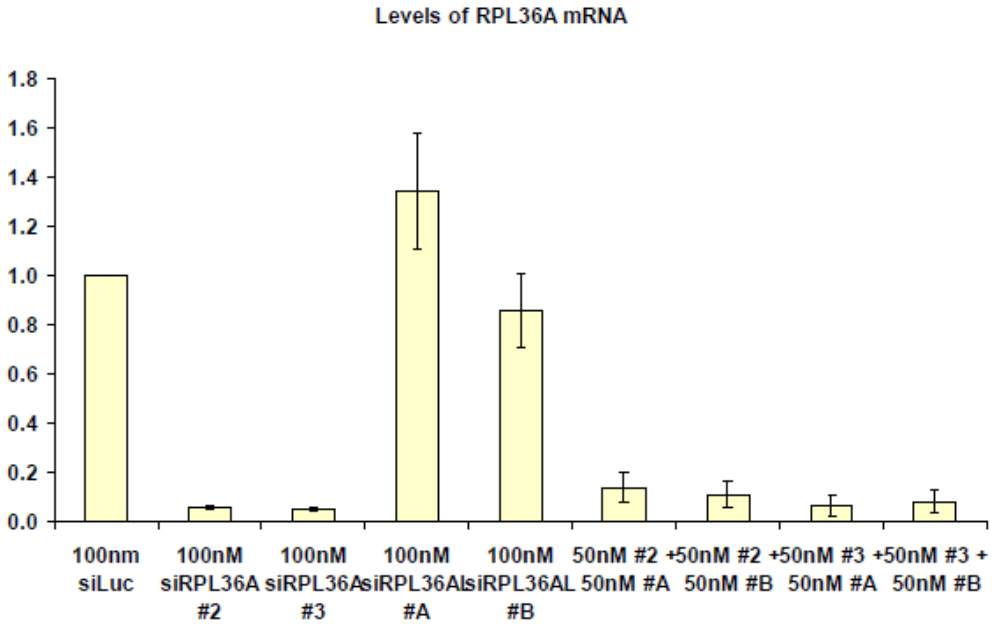

Single transfections
Flag-Mdm2
Myc-RPL36A
Double transfection
Flag-Mdm2 + Myc-RPL36A

FIGURE 3.S4

A


Levels of RPL36A mRNA
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
100nm siLuc
100nM siRPL36A #2
100nM siRPL36A #3
100nM siRPL36AL #A
100nM siRPL36AL #B
50nM #2 #A
+50nM #2 50nM #B
+50nM #3 50nM #A
+50nM #3 + 50nM #B

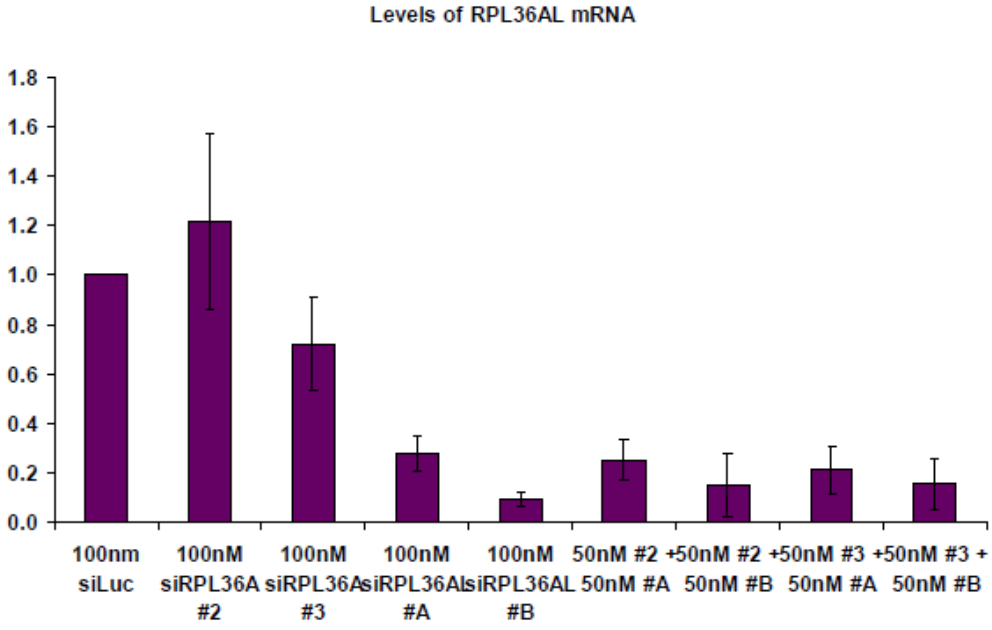

Levels of RPL36AL mRNA
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
100nm siLuc
100nM siRPL36A #2
100nM siRPL36A #3
100nM siRPL36AL #A
100nM siRPL36AL #B
50nM #2 #A
+50nM #2 50nM #B
+50nM #3 50nM #A
+50nM #3 + 50nM #B

FIGURE 3.S5

A

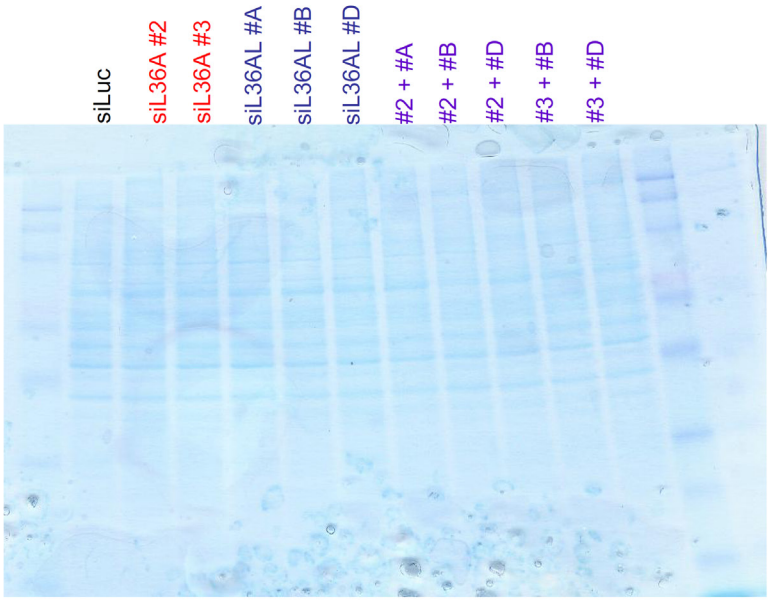

siLuc
siL36A #2
siL36A #3
siL36AL #A
siL36AL #B
siL36AL #D
#2 + #A
#2 + #B
#2 + #D
#3 + #B
#3 + #D

FIGURE 3.S6

A

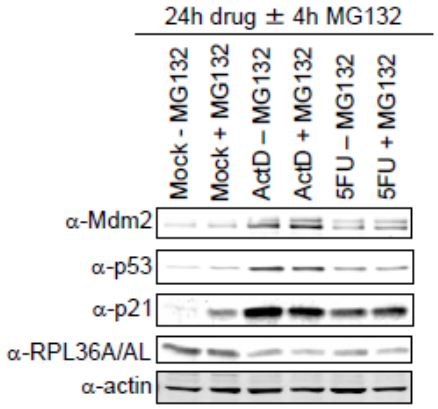
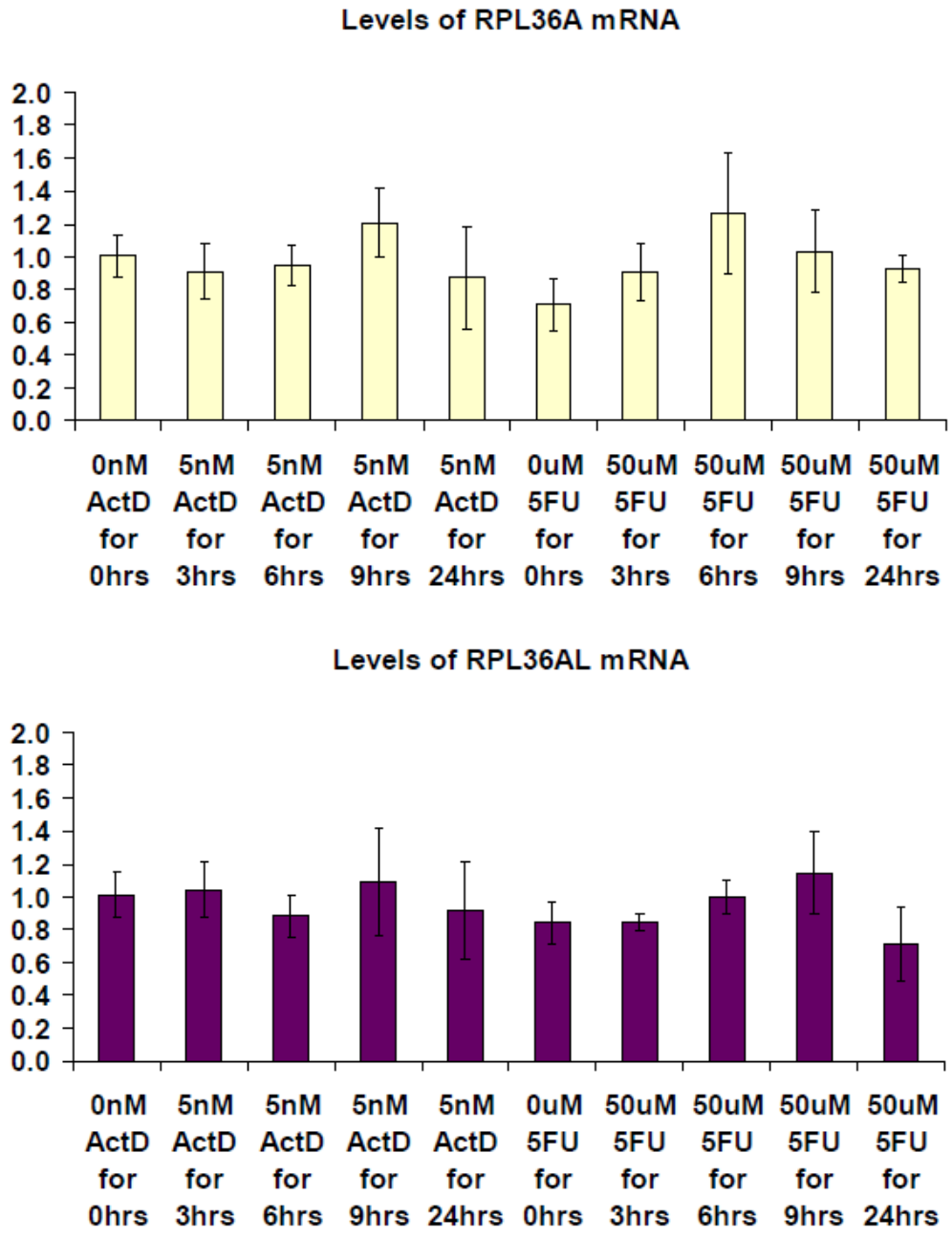

24h drug ± 4h MG132
Mock - MG132
Mock + MG132
ActD – MG132
ActD + MG132
5FU – MG132
5FU + MG132
α-Mdm2
α-p53
α-p21
α-RPL36A/AL
α-actin

FIGURE 3.S7

A


Levels of RPL36A mRNA
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0nM ActD for 0hrs
5nM ActD for 3hrs
5nM ActD for 6hrs
5nM ActD for 9hrs
5nM ActD for 24hrs
0uM 5FU for 0hrs
50uM 5FU for 3hrs
50uM 5FU for 6hrs
50uM 5FU for 9hrs
50uM 5FU for 24hrs
Levels of RPL36AL mRNA
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0nM ActD for 0hrs
5nM ActD for 3hrs
5nM ActD for 6hrs
5nM ActD for 9hrs
5nM ActD for 24hrs
0uM 5FU for 0hrs
50uM 5FU for 3hrs
50uM 5FU for 6hrs
50uM 5FU for 9hrs
50uM 5FU for 24hrs

CHAPTER 4

FUTURE DIRECTIONS

UNANSWERED QUESTIONS

Ribosomal proteins L37, S15, and S20

In Chapter 2, we demonstrated that like several other "effector" ribosomal proteins (RPs), RPL37, RPS15, and RPS20 can bind to Mdm2, inhibit its E3 ubiquitin ligase activity towards itself and p53, causing an increase in Mdm2 and p53 levels. As a result, select p53 targets become transcriptionally activated and cell cycle arrest and cell death are increased. Additionally, while the effect of most other "effector" RPs on MdmX is unknown, we were able to demonstrate RPL37, RPS15, and RPS20 can decrease its levels. Only RPL11 has been shown to similarly downregulate MdmX levels by enhancing Mdm2-mediated degradation (Gilkes et al., 2006); like RPL37, RPL11 cannot bind to MdmX directly. Therefore, we performed an *in vivo* ubiquitination assay to determine if RPL37, RPS15, or RPS20 can also enhance Mdm2-mediated degradation of MdmX. The data is preliminary, but it suggests RPL37 may be able to enhance Mdm2-mediated ubiquitination of MdmX (Figure 4.1a). I*n vivo* degradation assays also show RPS20 can enhance Mdm2-mediated degradation of MdmX (Figure 4.1b). It remains unclear how RPS15 regulates MdmX protein levels, and how RPL37 regulates MdmX mRNA levels.

The other “effector” RPs that have been shown to interact with Mdm2 also share an additional feature – they are necessary for a full response to ribosomal stress and their ablation by siRNA leads to an attenuation of the p53 response. On the other hand, “detector” RPs indirectly upregulate p53 levels when knocked down by siRNA via RPL11. Notably, even though RPS14 is an “effector” RP that decreases p53 levels under stressed conditions, in the absence of stress, it increases p53 levels in an RPL11-dependent manner (Zhou et al., 2013). Preliminary data suggests RPS15 may behave the same way (Figure 4.2a), and preliminary qRT-PCR data also suggests siRNA directed against either RPL37 or RPS15 may upregulate p53 targets promiscuously, while overexpression was selective (Figure 4.3a-b).

Thus, RPL37, RPS15, and RPS20 may not fit into the existing model of “effector” and “detector” RPs. In the absence of other stress, just their ablation by siRNA appears to lead to a defect in ribosomal biogenesis and upregulated levels of p53. It remains to be seen if their ablation by siRNA attenuates to response to ribosomal stress or not.

Ribosomal proteins L36A and L36AL

In Chapter 3, we demonstrated a novel function for RPL36A as a “repressor” of the Mdm2-p53 axis. RPL36A interacts with Mdm2 and stimulates its E3 ubiquitin ligase activity to decrease levels of p53. Preliminary data suggests that RPL36AL may behave like RPL36A, which make sense given their

amino acid sequences are 99% identical (Figure 4.4a-d). Additionally, unpublished data shows RPL12 may be another “repressor” RP, suggesting “repressors” may be a class of RPs with multiple members like “effectors” and “detectors” (Figure 4.4e-f).

siRNA against RPL36A or RPL36AL leads to an upregulation in p53 levels, which phenocopies “detector” RPs that do the same thing via RPL11. We hypothesized that siRPL36A or siRPL36AL differs from these other “detector” RPs in that ribosomal biogenesis is not disrupted so long as the other one is available to become part of the ribosome. But this argument would be strengthened if no change was observed in siRPL36A or siRPL36AL polysome profiles. Also, a lack of disruption to global protein synthesis by siRPL36A or siRPL36AL could be confirmed with a S^{35}-methionine pulse-chase experiment.

Additionally, unpublished data indicates RPL11 may be a confounding issue for the upregulation of p53 by siRPL36A or siRPL36AL. p53 levels are no longer elevated by siRPL36A or siRPL36AL in the absence of RPL11 (Figure 4.5a), suggesting regulation of p53 levels may be at least partially dependent upon RPL11. On the other hand, although siRPL36A or siRPL36AL do not appear to affect levels of RPL11, a double knockdown of RPL36A and RPL36A appears to reduce levels of RPL11 (Figure 4.5b). This suggests the upregulation of p53 by siRPL36A and siRPL36AL may not be dependent upon RPL11, since a drop in RPL11 levels mediated by siRNA causes p53 levels to drop. It is likely that ribosomal biogenesis is being disrupted by the reduction of both RPL36A

and RPL36AL, and levels of other 40S RPs are being reduced as a consequence.

Perhaps the answer may be that there are multiple pathways for RPL36A to downregulate p53 levels. For example, RPL26 is an “effector” that has been shown to upregulate p53 levels both by binding Mdm2 and inhibiting Mdm2-mediated degradation of p53, and by binding to p53 mRNA and augmenting its translation (Takagi et al., 2005; Chen and Kastan, 2010; Ofir-Rosenfeld et al., 2008; Zhang et al., 2010). The RPL11-dependent mechanism suggested by the siRNA experiments may function through Mdm2, while preliminary data from an experiment with Nutlin suggests a Mdm2-indepdendent mechanism may exist. Nutlin is a drug that prevents Mdm2 from interacting with p53, allowing them to be stabilized, but ectopic Myc-RPL36A is still able to decrease p53 levels in its presence (Figure 4.6a).

Finally, the data presented in Chapter 3 suggests that RPL36A relocalizes from a soluble state into an insoluble state in response to ribosomal stress. While this may not be a unique phenotype, a preliminary fractionation experiment suggests that unlike other RPs, a subset of cellular RPL36A and RPL36AL exists outside the ribosome in the absence of stress (Figure 4.7a). The hypothesis that RPL36A and RPL36AL relocalizes in response to stress, and this is necessary for a full p53 response, would be strengthened if their localization, along with Mdm2 and p53, could be visualized by immunofluorescent microscopy.

Why do so many ribosomal proteins interact with Mdm2?

A growing number of RPs have been shown to impact p53 levels, either directly or indirectly. Both 40S RPs and 60S RPs have been implicated, and at first glance, there is no obvious sequence motif common to the RPs that have been shown to regulate p53 levels and those that haven't. It is increasingly becoming important in the field to ascertain why so many RPs play seemingly redundant roles in the regulation of p53 levels.

One possible explanation for the proliferation of RPs that can regulate the Mdm2-p53 axis is that different RPs may activate p53 with different kinetics upon stress. For example RPS7 seems to act early, regulating the response to ActD and 5FU around 6 hours following drug treatment (Zhu et al., 2009), while RPL11 seems to be important later, around 24 hours after drug treatment (Zhang et al., 2003). Also, different RPs may regulate in p53 in some cell types while others are dominant in other cell types. For example, RPL26 was shown to upregulate p53 in MCF7 cells (Takagi et al., 2005) while ectopic RPL11 has been shown to increase p53 levels in U2OS cells (Lohrum et al., 2003). It is also possible that multiple RPs can cooperate to activate p53 maximally in response to stress. For example, one paper showed that overexpression of both RPL5 and RPL11 is much better than either one alone at stimulating p53 (Horn and Vousden, 2008). It remains to be seen which "effector" RPs can synergize and which cannot.

Finally, there may be subtle differences between the "effector" RPs in how they regulate p53. For example, RPS26 affects acetylation of p53 following

ribosomal stress but not phosphorylation (Cui et al., 2013). Thus, the various "effector" RPs may be able to cause different p53 targets to be upregulated, and lead to different cellular outcomes. For example, of 8 different p53 target genes, ectopic RPL37 can only upregulate p21 and Puma, while RPS15 and RPS20 were able to upregulate additional targets (Daftuar et al., 2013). Additionally, RPL37, RPS15, and RPS20 stimulate G2 arrest while RPL23, RPS7 and RPS25 have been shown to stimulate G1 arrest (Dai et al., 2004; Zhu et al., 2009; Zhang et al., 2013).

It should be noted that it is possible RPL5 and RPL11 may have additional extra-ribosomal functions beyond the roles that other "effector" RPs have in the RP-Mdm2-p53 pathway. Within the realm of ribosomal biogenesis, they have been shown to pre-assemble with 5S rRNA before being added to the 60S large ribosomal subunit (Steitz et al., 1988; Zhang et al., 2007). One recent publication found that RPL5 and RPL11 are protected from proteasomal degradation following ribosomal stress (Bursac et al., 2012). Finally, a tumor-derived mutant of Mdm2, Mdm2-C305F fails to co-immunoprecipitate with RPL5 and RPL11, and a mouse model exhibits accelerated Eμ-Myc driven lymphomagenesis (Lindstrom et al., 2007; Macias, 2010). More research needs to be done to demonstrate if other effector RPs also linger following ribosomal stress, or if they can interact with tumor-derived mutants of Mdm2.

CONCLUDING REMARKS

In this thesis, we identified novel extra-ribosomal functions for 4 different ribosomal proteins (RPs) in the Mdm2-p53 axis. The existence of an RP-Mdm2-p53 pathway has been uncovered over the past decade, and we have added to the complexity of this pathway, particularly with the identification of RPL36A as a "repressor" of it. Data from cells suggest activation of p53 by RPs is essential for a proper response to ribosomal stress, and animal models and human "ribosomopathies" also suggest RPs may function as haploinsufficient tumor suppressors *in vivo* (Amsterdam et al., 2004; Narla and Ebert, 2010). On the other hand, activation of p53 by the loss of an allele of an RP appears to be problematic for erythrocyte proliferation, and perhaps our finding that RPL36A suppresses the pathway could be exploited to prevent this pathology.

MATERIALS AND METHODS

Other materials and methods are described in Chapters 2 and 3.

Reagents

Immunoblots for RPL11 were performed using a commercial mouse monoclonal antibody (3A4A7, Zymed). Nutlin-3a was prepared in DMSO and stored at room temperature until use (Sigma). siRNA targeting RPL11 was previously reported (Jin et al 2004); the target sequence is 5'-GCAUUGGUAUCUACGGCCU-3'. The siRPS15 sequence used in Figures 4.2 and 4.3 targets 5'-ACCTACAAGCCCGTAAAGCA-3'. Other reagents are described in Chapters 2 and 3.

Ubiquitination assay

Assays for ubiquitinated MdmX were adapted from (Xirodimas et al, 2001). Briefly, H1299 cells were transfected with His-Ubiquitin, HA-MdmX, Flag-Mdm2, and Myc-RPs as indicated. 18 hours after transfection, H1299 cells were treated with 25 μM MG132 (Calbiochem) for 6 hours. Equivalent amounts of clarified cell lysates were incubated with 6M guanidine and pre-blocked Protein G Sepharose beads (GE Healthcare) then washed with 8M urea. His-proteins were eluted with 200 mM imidazole and prepared for gel electrophoresis and immunoblotting as described.

Cellular fractionation

Cellular fractionation was adapted from (Bursac et al, 2012). Briefly, U2OS cells were washed then lysed in low salt buffer (10 mM Hepes-NaOH pH 7.5, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA) supplemented with 0.3% NP-40 and 0.2% sodium deoxycolate. Nucleoplasmic and cytoplasmic fractions were separated by centrifugation at 2,800 g for 5 minutes. The resulting nucleoplasmic pellet was washed in low salt buffer before lysing with high salt buffer (10 mM Tris-HCl pH 7.2, 500 mM NaCl, 50 mM $MgCl_2$, 0.1 mM $CaCl_2$) supplemented with 50 U DNase I. The nucleoplasm was then centrifuged through a sucrose cushion (20% sucrose, 20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 300 mM KCl, 0.5% NP40) at 149,000 g for 2 hours to separate the nuclear ribosomal pellet from the nuclear non-ribosomal supernatant. The non-ribosomal nuclear fraction concentrated by trichloroacetic acid (TCA, Sigma) precipitation prior to immunoblotting.

REFERENCES

Amsterdam, A., Sadler, K.C., Lai, K., Farrington, S., Bronson, R.T., Lees, J.A., and Hopkins, N. (2004). Many ribosomal protein genes are cancer genes in zebrafish. PLoS biology *2*, E139.

Bursac, S., Brdovcak, M.C., Pfannkuchen, M., Orsolic, I., Golomb, L., Zhu, Y., Katz, C., Daftuar, L., Grabusic, K., Vukelic, I*., et al.* (2012). Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. Proceedings of the National Academy of Sciences of the United States of America *109*, 20467-20472.

Chen, J., and Kastan, M.B. (2010). 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes & development *24*, 2146-2156.

Cui, D., Li, L., Lou, H., Sun, H., Ngai, S.M., Shao, G., and Tang, J. (2013). The ribosomal protein S26 regulates p53 activity in response to DNA damage. Oncogene.

Daftuar, L., Zhu, Y., Jacq, X., and Prives, C. (2013). Ribosomal proteins RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network. PLoS one.

Dai, M.S., Zeng, S.X., Jin, Y., Sun, X.X., David, L., and Lu, H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Molecular and cellular biology *24*, 7654-7668.

Gilkes, D.M., Chen, L., and Chen, J. (2006). MDMX regulation of p53 response to ribosomal stress. The EMBO journal *25*, 5614-5625.

Horn, H., and Vousden, K. (2008). Cooperation between the ribosomal proteins L5 and L11 in the p53 pathway. Oncogene *27*, 5774-5784.

Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Molecular and cellular biology *24*, 7669-7680.

Lindstrom, M.S., Jin, A., Deisenroth, C., White Wolf, G., and Zhang, Y. (2007). Cancer-associated mutations in the MDM2 zinc finger domain disrupt ribosomal protein interaction and attenuate MDM2-induced p53 degradation. Molecular and cellular biology *27*, 1056-1068.

Lohrum, M.A., Ludwig, R.L., Kubbutat, M.H., Hanlon, M., and Vousden, K.H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer cell *3*, 577-587.

Macias, E., Jin, A., Deisenroth, C., Bhat, K., Mao, H., Lindstrom, M.S., and Zhang, Y. (2010). An ARF-independent c-MYC-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 Interaction. Cancer cell *18*, 231-243.

Narla, A., and Ebert, B.L. (2010). Ribosomopathies: human disorders of ribosome dysfunction. Blood. *115*, 3196-3205.

Ofir-Rosenfeld, Y., Boggs, K., Michael, D., Kastan, M.B., and Oren, M. (2008). Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. Molecular cell *32*, 180-189.

Steitz, J.A., Berg, C., Hendrick, J.P., La Branche-Chabot, H., Metspalu, A., Rinke, J., and Yario, T. (1988). A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. J Cell Biol. *106*, 545-556.

Takagi, M., Absalon, M.J., McLure, K.G., and Kastan, M.B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell *123*, 49-63.

Xirodimas, D., Saville, M.K., Edling, C., Lane, D.P., and Laín, S. (2001). Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 in vivo. Oncogene. 20, 4972-4983.

Zhang, J., Harnpicharnchai, P., Jakovljevic, J., Tang, L., Guo, Y., Oeffinger, M., Rout, M.P., Hiley, S.L., Hughes, T., and Woolford, J.L. Jr. (2007). Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes. Genes Dev. *21*, 2580-2592.

Zhang, Y., Wolf, G.W., Bhat, K., Jin, A., Allio, T., Burkhart, W.A., and Xiong, Y. (2003). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Molecular and cellular biology *23*, 8902-8912.

Zhang, Y., Wang, J., Yuan, Y., Zhang, W., Guan, W., Wu, Z., Jin, C., Chen, H., Zhang, L., Yang, X.*, et al.* (2010). Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26. Nucleic acids research *38*, 6544-6554.

Zhang, X., Wang, W., Wang, H., Wang, M.H., Xu, W., and Zhang, R. (2013). Identification of ribosomal protein S25 (RPS25)-MDM2-p53 regulatory feedback loop. Oncogene *32*, 2782-2791.

Zhou, X., Hao, Q., Liao, J., Zhang, Q., and Lu, H. (2013a). Ribosomal protein S14 unties the MDM2-p53 loop upon ribosomal stress. Oncogene *32*, 388-396.

Zhu, Y., Poyurovsky, M.V., Li, Y., Biderman, L., Stahl, J., Jacq, X., and Prives, C. (2009). Ribosomal protein S7 is both a regulator and a substrate of MDM2. Molecular cell *35*, 316-326.

FIGURE LEGENDS

Figure 4.1. RPL37 and RPS20 may enhance Mdm2-mediated ubiquitination and degradation of MdmX. (a) Preliminary data suggests possible enhancement of Mdm2-mediated ubiquitination of MdmX by RPL37. H1299 cells were seeded in 60mM tissue culture plates and transfected with His-Ubiquitin (4.0 μg), HA-MdmX (0.6 μg), Flag-Mdm2 (4.0 μg), and Myc-RPs (6.0 μg for RPL11; 1.2 μg for RPL37). 25 μM MG132 was added for 6 hours, and ubiquitinated MdmX species were assayed by isolating His-ubiquitin species and immunoblotting with α-MdmX. Inputs and IPs were run on separate gels. **(b) RPS20 enhances Mdm2-mediated degradation of MdmX.** U2OS cells were transfected with Flag-Mdm2 (2.0 μg), HA-MdmX (0.01 μg), and Myc-RPS20 (1.0 – 3.0 μg). Ectopic Mdm2, MdmX, and RPS20 were detected by immunoblotting with α-Flag, α-MdmX, and α-Myc. Immunoblots in panel (b) are taken from the same gel.

Figure 4.2. Activation of p53 by siRPS15 may be dependent upon RPL11. (a) Preliminary data suggests possible role for RPL11 in siRPS15-mediated p53 upregulation. U2OS cells were transfected with siRNA targeting RPL11 (50 nM), RPS15 (150 nM), or both. Cells were harvested and lysates were subjected to immunoblotting with the relevant antibodies.

Figure 4.3. siRNA against RPL37 or RPS15 may activate multiple p53 targets. (a-b) Preliminary data suggests siRNA against RPL37 or RPS15 may activate more targets than overexpression of RPs. U2OS cells were transfected with increasing amounts of siRNA (0 nM, 50 nM, 100 nM, 200 nM). Relative expression of each gene was determined in triplicate by quantitative RT-PCR and normalized to GAPDH.

Figure 4.4. RPL36AL and RPL12 may downregulate p53. (a) Preliminary data suggests RPL36AL may interact with acidic region of Mdm2 like RPL36A does. H1299 cells were transfected with Myc-RPL36AL (1.2 μg), Flag-Mdm2 full length (1.2 μg), Flag-Mdm2 truncation 1-220 (1.2 μg), Flag-Mdm2 deletion 222-272 (1.2 μg), Flag-Mdm2 deletion 222-340 (1.2 μg), Flag-Mdm2 deletion 340-437 (1.2 μg), and Flag-Mdm2 truncation 436-482 (1.2 μg). Myc-RPL36AL was immunoprecipitated with α-Myc and co-immunoprecipitation of each Mdm2 construct was assayed by immunoblotting with α-Flag. Inputs and IPs were run on separate gels. **(b-c) Preliminary data suggests RPL36AL may downregulate p53 levels like RPL36A and downregulate p53 targets.** U2OS cells were seeded in 60 mM tissue culture plates and transfected with increasing amounts of Myc-RPL36A (0 - 7.5 μg). Cells were harvested and split into fractions for immunoblot and quantitative RT-PCR. Lysates were subjected to immunoblotting with the relevant antibodies. Relative expression of each gene was also determined in triplicate by quantitative RT-PCR and normalized to

GAPDH. **(d) Preliminary data suggests RPL36AL may enhance Mdm2-mediated ubiquitination of p53 like RPL36A does.** H1299 cells were seeded in 60 mM tissue culture plates and transfected with HA-Ubiquitin (3.0 μg), p53 (0.75 μg), Flag-Mdm2 (7.5 μg), and Myc-RPL36AL (9.0 – 18.0 μg). MG132 was added for 6 hours, and ubiquitinated p53 species were assayed by immunoprecipitating with a-p53 and immunoblotting with α-HA. Inputs and IPs were run on separate gels. **(e) RPL12 interacts with Mdm2.** H1299 cells were transfected with Myc-RPL12 (1.2 μg), Flag-Mdm2 (1.2 μg), or both. (0.1 μg GFP was added as a control for transfection efficiency.) MG132 was added for 6 hours, and Myc-RPL12 was immunoprecipitated with α-Myc and co-immunoprecipitation of Flag-Mdm2 was assayed by immunoblotting with α-Flag. **(f) RPL12 decreases levels of p53 protein.** U2OS cells were transfected with increasing amounts of Myc-RPL12 (0 – 3.0 μg). Endogenous proteins were detected by immunoblotting with the relevant antibodies.

Figure 4.5. Activation of p53 by siRPL36A or RPL36AL may be dependent upon RPL11. (a) siRNA-mediated knockdown of RPL11 suppresses siRPL36A-mediated p53 upregulation. SJSA cells were transfected with siRNA targeting RPL11 (50 nM), RPL36A (100 nM), RPL36AL (100 nM), or combinations thereof. **(b) Double knockdown of RPL36A and RPL36AL decreases levels of RPL11 while activating p53.** SJSA cells were transfected

with 100 nM siRNA targeting RPL36A, RPL36AL, or both. Cells were harvested and lysates were subjected to immunoblotting with the relevant antibodies.

Figure 4.6. RPL36A may also downregulate p53 independently of Mdm2. (a) RPL36A decreases levels of p53 in the presence of Nutlin. U2OS cells were transfected with empty vector (3.0 μg) or Myc-RPL36A (3.0 μg). 2 μM Nutlin was added to the culture medium for 6 hours before harvesting. Endogenous proteins were detected by immunoblotting with the relevant antibodies. Immunoblots are taken from the same gel.

Figure 4.7. RPL36A and RPL36AL may exist in a ribosomal-free fraction. (a) Preliminary data suggests RPL36A and RPL36A may exist unbound to ribosomes in the nucleoplasm. U2OS cells were treated with 5nM actinomycin D for 0 – 5 hrs then fractionated into ribosomal nucleoplasmic fractions and non-ribosomal nucleoplasmic fraction as described. Endogenous proteins were detected by immunoblotting with the relevant antibodies.

FIGURE 4.1

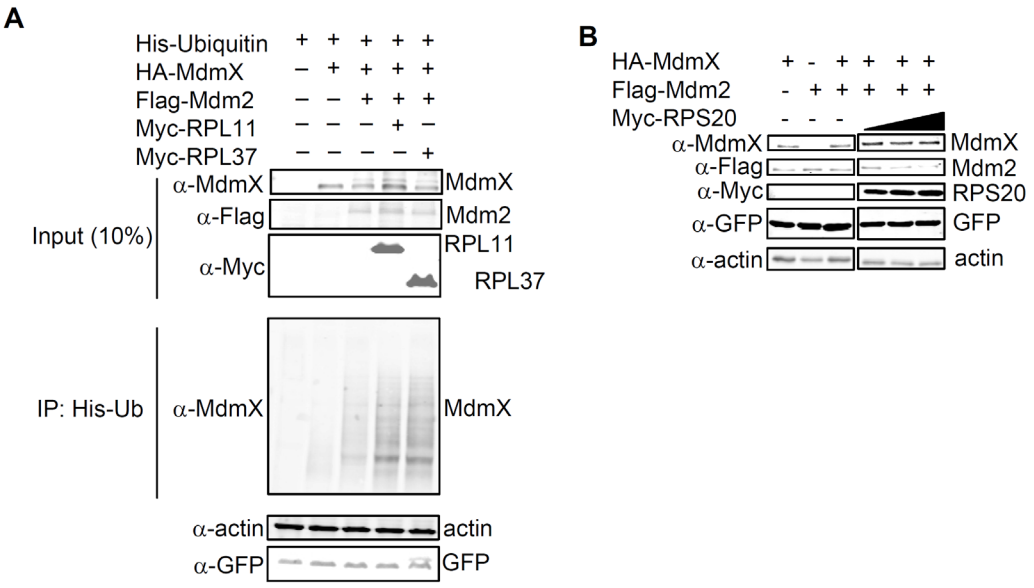

A
His-Ubiquitin + + + + +
HA-MdmX − + + + +
Flag-Mdm2 − − + + +
Myc-RPL11 − − − + −
Myc-RPL37 − − − − +
Input (10%)
α-MdmX
MdmX
α-Flag
Mdm2
α-Myc
RPL11
RPL37
IP: His-Ub
α-MdmX
MdmX
α-actin
actin
α-GFP
GFP
B
HA-MdmX + - + + + +
Flag-Mdm2 - + + + + +
Myc-RPS20 - - -
α-MdmX
MdmX
α-Flag
Mdm2
α-Myc
RPS20
α-GFP
GFP
α-actin
actin

FIGURE 4.2

A

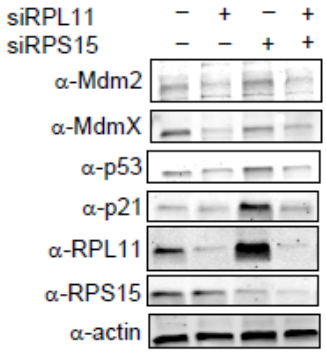
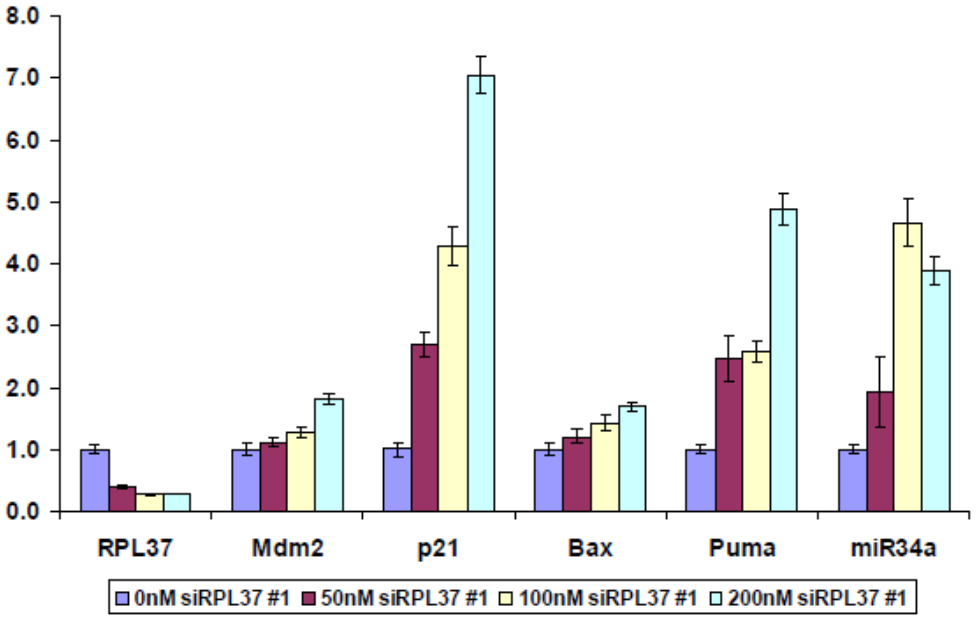

siRPL11 − + − +
siRPS15 − − + +
α-Mdm2
α-MdmX
α-p53
α-p21
α-RPL11
α-RPS15
α-actin

FIGURE 4.3

A


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
RPL37
Mdm2
p21
Bax
Puma
miR34a
0nM siRPL37 #1
50nM siRPL37 #1
100nM siRPL37 #1
200nM siRPL37 #1

B

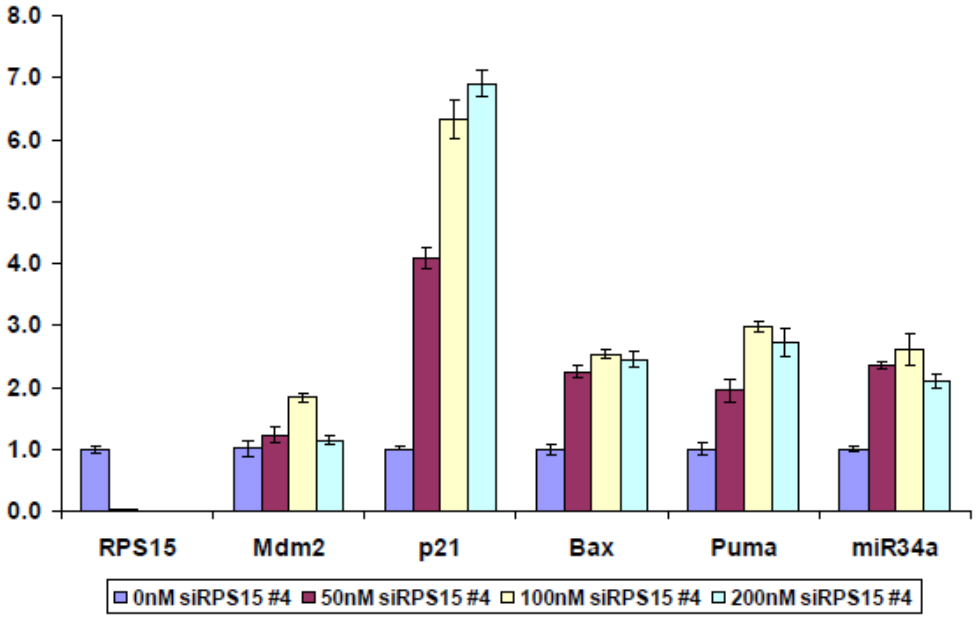

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
RPS15
Mdm2
p21
Bax
Puma
miR34a
0nM siRPS15 #4
50nM siRPS15 #4
100nM siRPS15 #4
200nM siRPS15 #4

FIGURE 4.4

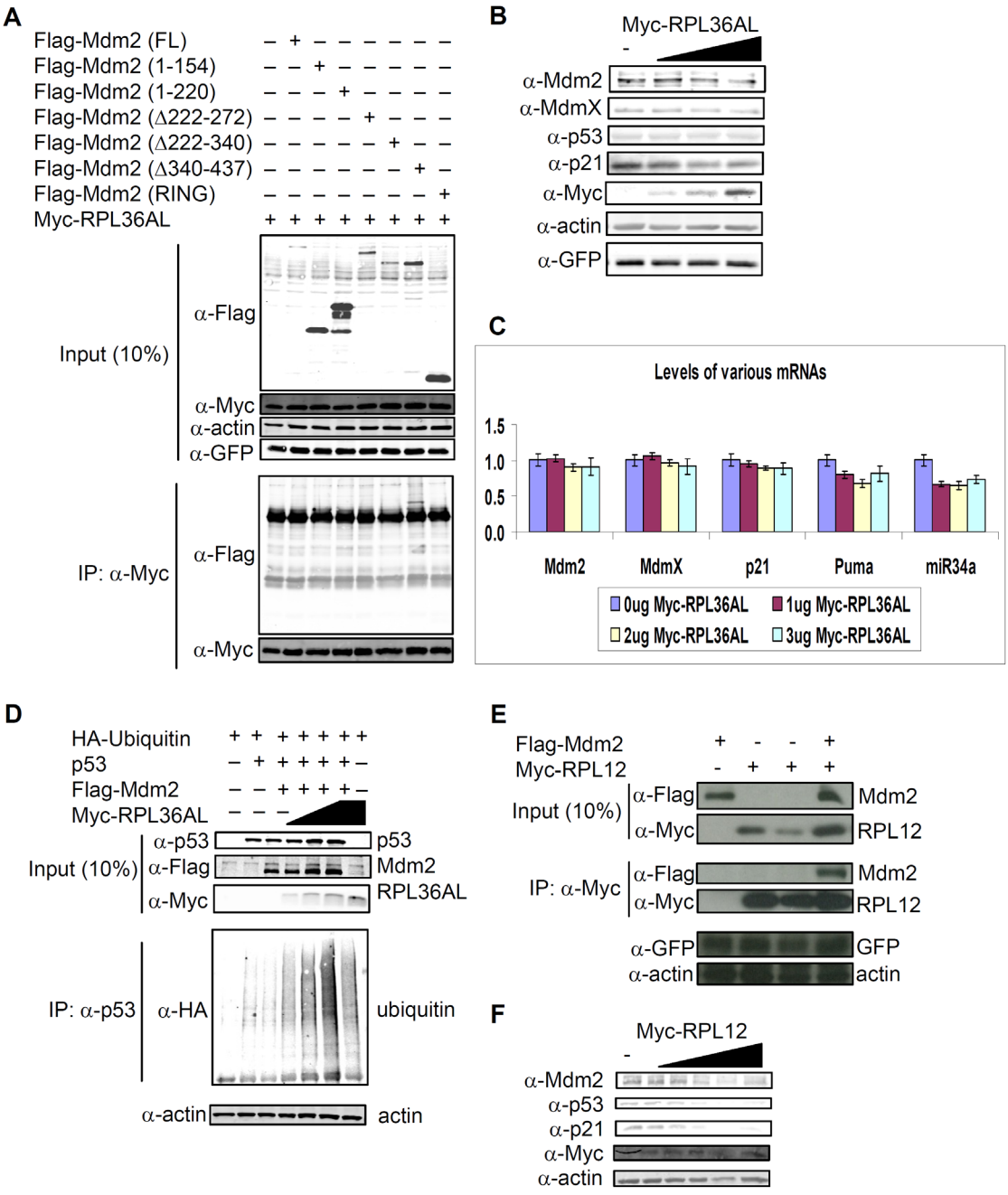

A
Flag-Mdm2 (FL)
Flag-Mdm2 (1-154)
Flag-Mdm2 (1-220)
Flag-Mdm2 (Δ222-272)
Flag-Mdm2 (Δ222-340)
Flag-Mdm2 (Δ340-437)
Flag-Mdm2 (RING)
Myc-RPL36AL
Input (10%)
α-Flag
α-Myc
α-actin
α-GFP
IP: α-Myc
α-Flag
α-Myc
B
Myc-RPL36AL
α-Mdm2
α-MdmX
α-p53
α-p21
α-Myc
α-actin
α-GFP
C
Levels of various mRNAs
1.5
1.0
0.5
0.0
Mdm2
MdmX
p21
Puma
miR34a
0ug Myc-RPL36AL
1ug Myc-RPL36AL
2ug Myc-RPL36AL
3ug Myc-RPL36AL
D
HA-Ubiquitin
p53
Flag-Mdm2
Myc-RPL36AL
Input (10%)
α-p53
α-Flag
α-Myc
p53
Mdm2
RPL36AL
IP: α-p53
α-HA
ubiquitin
α-actin
actin
E
Flag-Mdm2
Myc-RPL12
Input (10%)
α-Flag
α-Myc
Mdm2
RPL12
IP: α-Myc
α-Flag
α-Myc
Mdm2
RPL12
α-GFP
α-actin
GFP
actin
F
Myc-RPL12
α-Mdm2
α-p53
α-p21
α-Myc
α-actin

FIGURE 4.5

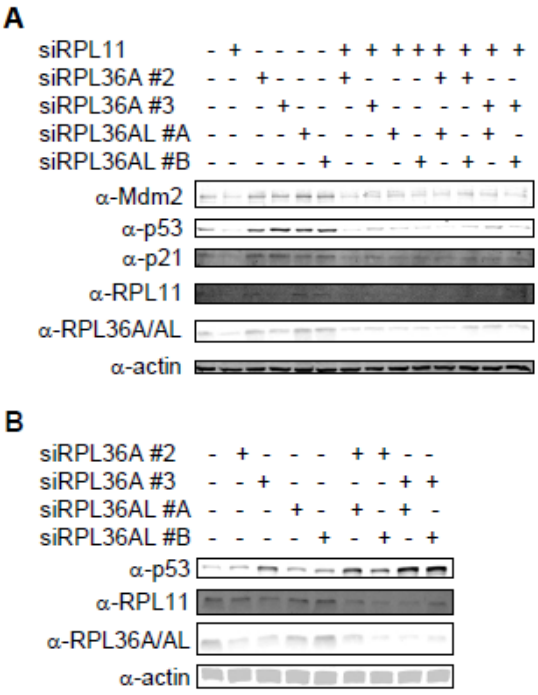

A
siRPL11 - + - - - - + + + + + + + +
siRPL36A #2 - - + - - - + - - - + + - -
siRPL36A #3 - - - + - - - + - - - - + +
siRPL36AL #A - - - - + - - - + - + - + -
siRPL36AL #B - - - - - + - - - + - + - +
α-Mdm2
α-p53
α-p21
α-RPL11
α-RPL36A/AL
α-actin
B
siRPL36A #2 - + - - - + + - -
siRPL36A #3 - - + - - - - + +
siRPL36AL #A - - - + - + - + -
siRPL36AL #B - - - - + - + - +
α-p53
α-RPL11
α-RPL36A/AL
α-actin

FIGURE 4.6

A

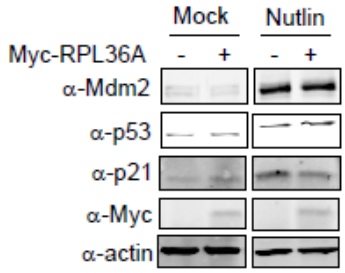
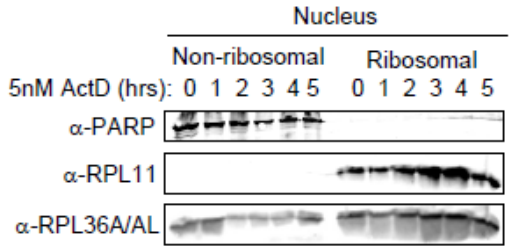


FIGURE 4.7

A


Nucleus
Non-ribosomal
Ribosomal
5nM ActD (hrs): 0 1 2 3 4 5 0 1 2 3 4 5
α-PARP
α-RPL11
α-RPL36A/AL